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In vivo and *in vitro* microdialysis sampling of free fatty acids

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Abstract

Microdialysis is a technique that allows continuous sampling of compounds from the interstitial fluid of different tissues with minimal influence on surrounding tissues and/or whole body function. In the present study, the feasibility of using microdialysis as a technique to sample free fatty acids (FFA) was investigated both *in vitro* and *in vivo*, by use of a high molecular weight (MW) cut-off membrane (3 MDa) and a push–pull system to avoid loss of perfusion fluid through the dialysis membrane. The relative recovery was examined *in vitro* for three different concentrations of radiolabelled oleic acid-BSA solutions (oleic acid:BSA molar ratio 1:1) and for various temperatures and flow rates. The recovery of oleic acid was found to be dependent on the concentration of analyte in the medium surrounding the membrane (17.3%, 29.0% and 30.6% for 50, 100 and 200 μM oleic acid-BSA solutions, respectively). Addition of 0.25% BSA to the perfusion fluid resulted, however, in a concentration-independent recovery of 31.4%, 28.1% and 28.1% for the 50, 100 and 200 μM solutions, respectively.

The capability of the method to measure FFA together with glycerol was investigated *in vivo* in visceral adipose tissue of rats, before and after lipolytic treatment with the β_3-adrenergic agent, BRL37344. BRL37344 caused an increase in both dialysate FFA and glycerol, although the increase was markedly higher for glycerol, amounting to 24.5% and 329.2% increase from baseline, respectively. Subsequent *in vitro* test of probe performance revealed a decrease in the dialysing properties with regard to FFA, but not glycerol. This suggests that clogging of the membrane pores after 110 min prevented the measurement of the full FFA response *in vivo*.

Keywords: Microdialysis; Fatty acids; Glycerol; Recovery; High molecular weight cut-off membrane

1. Introduction

In vivo microdialysis is a technique that allows continuous sampling from the interstitial fluid of different tissues with minimal influence on surrounding tissues and/or whole body function. A microdialysis probe is inserted in the tissue of interest and perfused at a constant flow rate with a physiological buffer. The tip of the probe consists of a semi permeable membrane through which compounds in the interstitial fluid of the tissue can diffuse and subsequently be sampled from the outlet tubing of the probe.

The technique has been used extensively to examine lipolytic activity by measuring glycerol release from adipose tissue [1–3]. Glycerol and FFA forms the end-products of lipolysis, e.g. hydrolysis of triacylglycerol. FFA can be re-utilized by adipose tissue through a re-esterification process, whereas glycerol is not re-utilized to any significant extent due to almost complete lack of the enzyme glycerol kinase in adipose tissue [4]. This absence of re-utilization combined with problems in sampling poorly water-soluble FFA by microdialysis, has made glycerol the analyte of choice for studies of lipolysis. However, released FFA not only serves as an important energy source being substrate for beta-oxidation it also seems to have great implications for both insulin action [5] and secretion [6–8]. This well established involvement of FFA in the development of insulin resistance has increased the focus on FFA considerably. Determination of FFA release by use of microdialysis might therefore provide a valuable tool for a better understanding of these events and a more comprehensive dynamic index of adipose tissue metabolism could be obtained by combined sampling of FFA and glycerol.

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However, the microdialysis technique is most suitable for small, hydrophilic compounds such as glycerol, which can easily pass through the membrane. Difficulties are on the other hand evident regarding recovery of lipophilic compounds due to: poor solubility in the aqueous perfusion fluid, adherence to the microdialysis devices and/or binding to proteins, which are too large to diffuse through the membrane. This is also true for FFA due to both albumin binding and their lipophilic nature [9].

The aim of the present study was to investigate the feasibility of sampling unbound as well as albumin-bound FFA, by use of a microdialysis membrane with a molecular weight cut-off high enough to allow albumin to pass through it. To characterize the system before implementation of the technique *in vivo* various parameters affecting FFA recovery were evaluated *in vitro*. Microdialysis probes were implanted in visceral adipose tissue of rats and FFA and glycerol were sampled before and after lipolytic stimulation by the β_3-adrenergic agonist, BRL37344.

2. Materials and methods

2.1. Chemicals

Radioactive [9,10-^{3}H(N)]-oleic acid, specific activity = 26.3 Ci/mmol, and [^{14}C(U)]-glycerol, specific activity = 151 mCi/mmol, were obtained from Perkin-Elmer Life Sciences (Boston, MA). BRL37344, oleic acid, glycerol and bovine serum albumin (BSA), essentially fatty acid free, were all purchased from Sigma (St. Louis, MO). Ringer's perfusion fluid, T1, for peripheral tissues was from CMA/Microdialysis (Stockholm, Sweden). Ecoscint A scintillation liquid was obtained from National Diagnostics (Atlanta, GA).

2.2. Animals

Four female wistar rats weighing 400–500 g (Taconic, Lille Skensved, Denmark) were housed two and two in conventional rat cages. The rats were subjected to a 12-h dark:12-h light cycle and kept at a constant room temperature of 20–22 °C. The animals had free access to standard chow and water.

2.3. Preparation of radiolabelled sample solutions

Oleic acid was used as a model FFA compound since it is the most abundant FFA in plasma [10]. Radiolabelled oleic acid solutions were prepared by evaporating ethanol from a mixture of ^{3}H-oleic acid and non-labelled oleic acid. Ringer's solution and BSA were then added to give final oleic acid concentrations of: 50, 100 and 200 μM, keeping a constant molar ratio of 1:1 between oleic acid and BSA. A radiolabelled 100 μM glycerol solution was prepared in a similar way. A high glycerol recovery is well established, but was nevertheless tested to confirm the feasibility of sampling glycerol with this particular microdialysis set-up and to determine the *in vitro* recovery.

2.4. Microdialysis system

Microdialysis was performed using commercial available polyethylene MAB 5 probes (Microbiotech AB, Stockholm, Sweden) with a membrane length of 10 mm and a 3 MDa molecular weight cut-off. The inlet tubing of the microdialysis probe was connected to an infusion pump (Harvard Apparatus, model 11, Holliston, MA) perfusing the probe with Ringer's solution at a constant speed. A peristaltic pump (Gilson, Minipuls 3, Middleton, WI) was connected to the outlet tubing in order to prevent perfusion fluid loss from the probe, by pulling the fluid through the tubing (see Fig. 1). The actual flow rates of the two pumps were determined individually without having a probe connected. The sample tubes were weighed before and after sampling for a given time period and the flow rate was calculated. The pumps were then set to comparable flow rates. After connecting the probe, the flow rate for the whole system was tested again by weighing the sample tubes in order to assure that no loss of perfusion fluid took place. Prior to use, the microdialysis probes were prepared according to the manufacture's instructions. The probes were rinsed thoroughly and stored in clean, distilled water after use. Probes for *in vitro* experiments were used several times, whereas a new probe was used for every *in vivo* experiment.

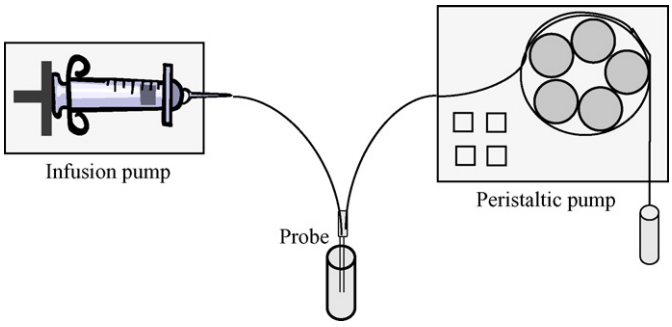

Fig. 1. Schematic drawing of the microdialysis set-up. The microdialysis probes were perfused with perfusion fluid via an infusion pump. A peristaltic probe was connected to the outlet tubing of the probe in order to pull the fluid through the system. The probe was implanted in the mesenteric adipose tissue of anaesthetized rats.

2.5. In vitro microdialysis experiments

The probe was placed in a 1.5 ml Eppendorf vial containing the sample solution of interest and perfused with perfusion fluid. The system was allowed to equilibrate for at least 20 min after elimination of any dead volume in the tubing. Samples were subsequently collected from the outlet tubing at 15 min intervals for minimum 1 h. Each type of experiment was tested for 2 h, at least once, to verify a constant recovery over a longer time period and was performed using 2–3 different probes to ensure that the results were independent of the particular probe. Unless otherwise stated, all tests were performed at ambient room temperature with a flow rate of 1 μl/min. The content of ^{3}H-oleic acid or ^{14}C-glycerol in the samples was measured by liquid scintillation. Relative recovery (%) was calculated as the concentration of analyte in the dialysate relative to the actual concentration in the solution surrounding the probe. The effect on relative recovery was investigated for the parameters: concentration, flow rate and temperature as described below.

The concentration dependence of recovery was studied for three different concentrations of radiolabelled oleic acid-BSA

solutions: 50, 100 and 200 μM (oleic acid:BSA molar ratio 1:1). The FFA concentrations tested were in the physiological range, which has been reported to be 90 μM in the interstitial space [11]. The experiments were performed with or without 0.25% BSA added to the Ringer's perfusion fluid ($n = 3$–4). Furthermore, addition of 1% and 4% BSA to the perfusion fluid was tested.

Relative recovery of the radiolabelled 100 μM oleic acid-BSA solution was investigated at different flow rates: 0.5, 1 and 2 μl/min. Ringer's solution with 0.25% BSA was used as perfusion fluid ($n = 3$). Furthermore, in order to determine the relationship between relative recovery and temperature of the sample solution, experiments were performed at both ambient room temperature and with the vial immersed in either a 37 °C temperature controlled water bath or a 2.5 °C ice bath. These experiments were likewise conducted with the radiolabelled 100 μM oleic acid-BSA solution using Ringer's solution with 0.25% BSA as perfusion fluid ($n = 3$).

The relative recovery of glycerol was studied for a radiolabelled 100 μM glycerol-BSA solution (glycerol:BSA molar ratio 1:1). Perfusion fluid was Ringer's solution with 0.25% BSA and the flow rate was 1 μl/min ($n = 3$).

2.6. In vivo microdialysis experiments

On the day of experiment, one rat was anaesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg). A small 1–1.5 cm incision was made in the abdominal skin and peritoneum, and a microdialysis probe was then carefully inserted in the mesenteric adipose tissue. The mesenteric adipose tissue was chosen since it has previously been reported that lipolysis stimulated via the β-adrenergic system is higher in the visceral fat [12]. Correct implantation of the probe was ascertained by visual inspection. Analgesic treatment was given prior to operation in the form of Carprofen (5 mg/kg, subcutaneously). During the experiment body temperature was maintained with an electric blanket (CMA/150 Temperature Controller, CMA Microdialysis, Stockholm, Sweden). Supplementary injections of Pentobarbital were given as required to maintain anaesthesia.

The probe was perfused with Ringer's solution containing 0.25% BSA at a constant flow rate of 1 μl/min. After a 120 min equilibrium period, microdialysis sampling was initiated and dialysate were subsequently collected at 20 min intervals throughout the entire experiment. Since both FFA and glycerol were measured in the same dialysate a longer sampling time was required *in vivo* than *in vitro*. Following 70 min of baseline sampling, an intraperitoneal injection (1 mg/kg) of the specific β_3-adrenerg agonist, BRL37344, was given at time 0 min, in order to stimulate lipolysis. Plasma samples were taken at time: −70, −10, 10, 70, 110 and 150 min in EDTA-coated tubes containing NaF to prevent lipolysis during storage [13]. After termination of the experiment the probe was carefully removed and the rat was decapitated. All dialysate and plasma samples were stored at −20 °C and analysed for glycerol and FFA within 24 h by use of enzymatic kits (GPO trinder, Sigma and NEFA-C kit, Wako Chemicals GmbH).

The dialysing properties of the probes were determined before and after each experiment by placing the probes in a solution containing: 200 μM FFA, 200 μM glycerol and 200 μM BSA. The probes were perfused with Ringer's solution containing 0.25% BSA at a flow rate of 1 μl/min for 1 h. The relative recovery was then determined as previously described.

2.7. Statistical analysis

All data are presented as means ± S.E.M. Treatment means for the *in vitro* part were analysed by use of one-way ANOVA, where appropriate. In case of an overall significant treatment effect in the ANOVA, the Newman–Keuls test for multiple comparisons was applied to determine the differences between the individual treatments. *In vivo* comparisons were made using a two-tailed Student's paired *t*-test. $p < 0.05$ were considered as significant. GraphPad Prism Version 4.03 was used for all statistical calculations.

3. Results

3.1. In vitro microdialysis

The relative recovery was significantly lower ($p < 0.001$) for the radiolabelled 50 μM oleic acid-BSA solution (17.3 ± 1.3%) as compared to the 100 and 200 μM solutions (29.0 ± 1.0% and 30.6 ± 0.4%, respectively). No significant difference was seen between the recoveries of the 100 and 200 μM oleic acid-BSA solutions. Addition of 0.25% BSA to the Ringer's perfusion fluid resulted, however, in a concentration independent recovery for all the tested concentrations (31.4 ± 1.1%, 28.1 ± 1.5% and 28.1 ± 0.8% for the 50, 100 and 200 μM solutions, respectively). No significant difference was seen between the recoveries after addition of 0.25% BSA to the perfusion fluid and the recoveries for the 100 and 200 μM solutions without addition of 0.25% BSA (Fig. 2). Addition of 1% and 4% BSA to the Ringer's solution had no additional effect on recovery (data not shown).

The recovery of ^{3}H-oleic acid as a function of flow rate is shown in Fig. 3. The relative recovery decreased with increasing flow rates from 40.4 ± 0.9% at 0.5 μl/min to 14.9 ± 0.3% at 2 μl/min. No significant difference in relative recovery was found when varying the temperature (Fig. 4). At 2.5 °C, ambient room temperature and 37 °C the recoveries were 29.2 ± 1.3%, 28.1 ± 1.5% and 29.5 ± 0.9%, respectively. The relative recovery of the 100 μM glycerol solution was found to be 70.4 ± 0.8% (data not shown).

3.2. In vivo microdialysis

The *in vivo* results are shown in Figs. 5a, b and 6. The period between sampling start at time −70 min and the bolus injection of the β_3-adrenergic agent BRL37344 at time 0 min is considered as the baseline period. The mean baseline concentrations of FFA and glycerol were 65.84 ± 3.00 μM (range: 56.26–77.07 μM) and 62.14 ± 7.94 μM (range: 34.46–103.42 μM), respectively.

After the injection of BRL37344 both FFA and glycerol levels increased. The maximum percentage increase compared to the baseline was 24.5% at time 70 min for FFA (Fig. 5a) and

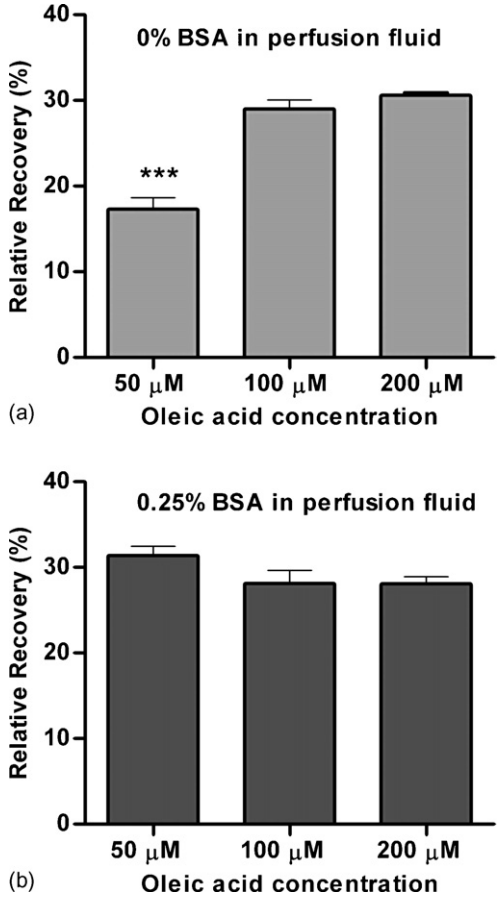

Fig. 2. Relative recovery (%) of FFA solutions: 50, 100 or 200 μM (radiolabelled oleic acid-BSA solutions). The perfusion fluid contained either 0% (a) or 0.25% BSA (b). All experiments were carried out at a flow rate of 1 μl/min and at room temperature. Values are mean ± S.E.M. $n = 3$–4; $^{***}p < 0.001$.

329.2% at time 90 min for glycerol (Fig. 5b). The mean response to the BRL37344 treatment for the period with constant recovery were significantly higher compared to the mean baseline concentration for both FFA and glycerol ($p = 0.043$ and 0.048, respectively).

The *in vitro* performance of the probes before and after the *in vivo* experiment was unaltered with respect to glycerol, while

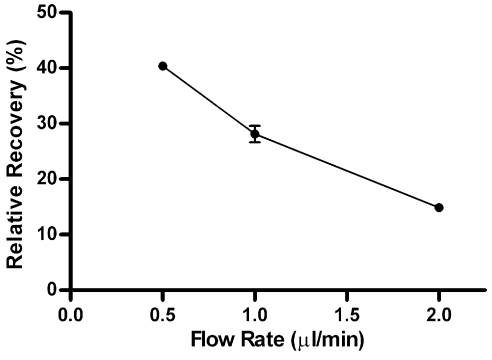

Fig. 3. The relationship between *in vitro* recovery and perfusate flow rate. The experiments were carried out in a radiolabelled 100 μM oleic acid-BSA solution with Ringer's solution included 0.25% BSA as perfusion fluid and at room temperature. Values are mean ± S.E.M. $n = 3$.

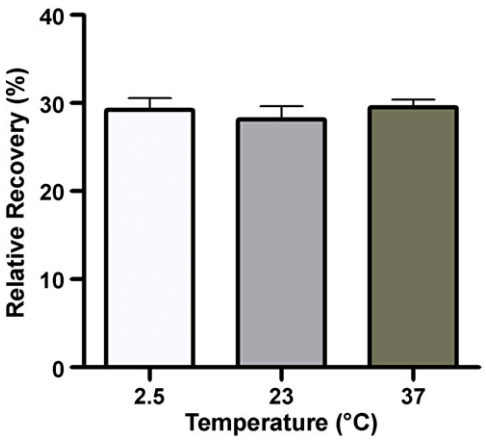

Fig. 4. The relationship between *in vitro* recovery and temperature of the sample medium. The experiments were carried out in a radiolabelled 100 μM oleic acid-BSA solution with Ringer's solution included 0.25% BSA as perfusion fluid. Flow rate 1 μl/min. Values are mean ± S.E.M. $n = 3$.

probe recovery of FFA markedly decreased from an average of 30.5% before the experiment to 16.3% after the experiment.

As a control of the systemic effectiveness of the adrenergic stimulation, plasma concentrations of FFA and glycerol were measured before and after BRL37344 treatment (Fig. 6). The mean baseline plasma concentration ranged from 357.05 to 763.00 μM for FFA and 164.72 to 413.01 μM for glycerol. Augmented lipolysis caused plasma levels to increase from the baseline, although glycerol more than FFA with maximal increments of 301% and 108%, respectively. The mean BRL37344

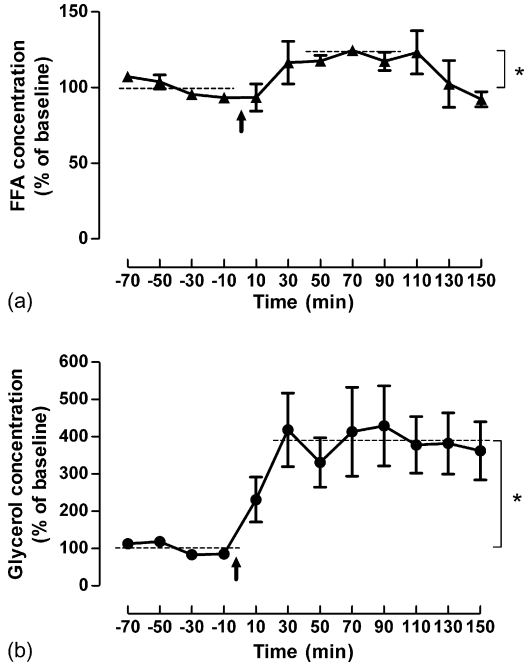

Fig. 5. Sampling of FFA (a) and glycerol (b) in the mesenteric adipose tissue before and after lipolytic stimulation by the β_3-adrenergic agent, BRL37344 (1 mg/kg, intraperitoneal) at time 0 min (arrow). Dialysate concentrations are expressed as percent of the mean baseline level. Values are mean ± S.E.M. $n = 3$–4. 100% = 56.26–77.07 μM for FFA (a) and 34.46–103.42 μM for glycerol (b). $^{*}p < 0.05$ compared with mean baseline.

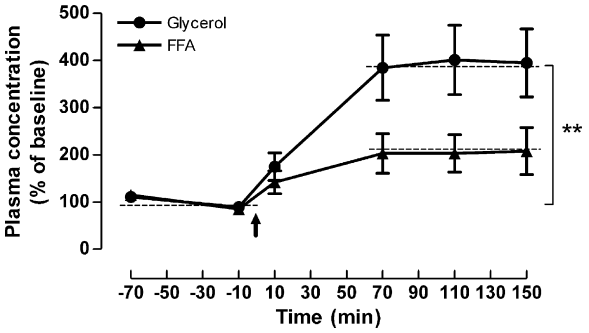

Fig. 6. Effect of the β_3-adrenergic agent BRL37344 (1 mg/kg, intraperitoneal) on plasma levels of FFA and glycerol. The arrow marks the administration of BRL37344. Plasma concentrations are expressed as percent of the mean baseline level. Values are mean ± S.E.M. $n = 3$–4. 100% = 357.05–763.00 μM for FFA and 164.72–413.01 μM for glycerol. $^{**}p < 0.01$ compared with mean baseline.

response was significantly higher than the mean baseline concentration for glycerol ($p = 0.0013$) although not for FFA.

4. Discussion

One of the objectives of the present study was to investigate the possibility of using a microdialysis probe with a high MW cut-off membrane 3 MDa to sample unbound as well as albumin-bound FFA. The 3 MDa membrane allows large molecules to pass through, in contrast to the conventional membranes with much lower cut-offs (usually 4 or 20 kDa). The disadvantage of the 3 MDa and other high MW cut-off membranes is, however, a high risk of loosing the perfusion fluid to the medium surrounding the probe due to back pressure in the outlet tubing [14]. This probably also explains why probes with a low MW cut-off is normally used. In the current study, the problem with perfusion fluid loss was overcome by using a push–pull system, where a peristaltic pump was connected to the outlet tubing in order to pull the perfusion media out of the probe.

Recovery of non-albumin bound FFA by use of microdialysis with a low cut-off probe has previously been attempted by Carneheim and Ståhle [9] reporting the *in vitro* recovery of an oleic acid solution to be about 5% by addition of 4% BSA to the perfusion fluid. However, subsequent efforts to measure the unbound FFA concentration *in vivo* did not succeed, as the unbound level was probably too small to be measurable, even after lipolytic stimulation by norepinephrine.

Since most of the FFA in the interstitial fluid is bound to albumin, as in the blood stream, the approach of the present study was therefore to sample both the unbound and the protein-bound fraction of FFA.

The *in vitro* data obtained from the current study showed a markedly higher FFA recovery as compared with the study by Carneheim and Ståhle [9]. In fact the improvement corresponds to a five-fold increase. This was probably due to the high cut-off membrane used in the present study as well as the longer tip, thus exposure of a greater surface area of the membrane to the tissue interstitium. When using perfusate without BSA, the recovery was concentration dependent. This may be explained by binding of oleic acid to the microdialysis material, which would be most pronounced at low concentrations as it might becomes saturated as the concentration increases. This particular problem has previously been observed by others. When performing microdialysis of lipophilic substances Groth and co-workers observed that the recovery of sodium fusidate increased with increasing concentrations, probably due to interaction between the membrane and sodium fusidate [13]. However, in our hands the addition of 0.25% BSA to the perfusion fluid resulted in a concentration independent recovery, indicating that some sort of binding took place without added BSA. Adding higher concentrations of BSA (1% and 4%) to the perfusion fluid did not improve the recovery additionally. The lowest BSA concentration was therefore chosen for further studies, in order to minimise interference with the sampling space surrounding the probe. In agreement with several previously reported microdialysis experiments [15–18] the recovery was found to decrease with increasing flow rates as the dilution of the dialysed analyte becomes greater at higher flow rates. The temperature of the medium surrounding the probe did not affect the recovery.

Sampling of labelled glycerol was conducted to demonstrate the feasibility of the current set-up for studies of both lipolytic end-products. The much higher recovery seen for glycerol was expected due to the smaller size of this analyte and hence a higher diffusion rate.

The very promising *in vitro* results led to further testing of the technique *in vivo*. The *in vivo* data confirmed the feasibility of using the technique for concomitant sampling of both FFA and glycerol as both analytes were present in the dialysate in measurable levels. Stimulation of lipolysis by the β_3-adrenergic agent, BRL37344, significantly increased both FFA and glycerol, although the increase was about 13-fold greater for glycerol. This should be seen in the light of the fact, that the recovery of glycerol, at least *in vitro*, is more than twice as good as the FFA recovery.

FFA has previously been sampled together with glycerol in human subcutaneous adipose tissue by use of the open-flow microperfusion technique [19]. Stimulation of lipolysis with the non-selective β-adrenergic agonist isoproterenol caused a concomitant increase in both glycerol and FFA compared to an adjacent control fat-pad of the same individual. Also in this study, the glycerol increase was greater than for FFA, although not as markedly as in the present study. The FFA increase was given as a pooled measure for 2.5 h and not as a dynamic index. The relative recovery of FFA by this set-up was not reported and likewise no characterization of the FFA sampling was described.

Testing the probe performance before and after the *in vivo* study, revealed a marked reduction in the FFA recovery from 30.5% to 16.3%, showing that the probe eventually at later time points became clogged. In agreement with this, a decrease in protein recovery over time has recently been described by Rosenbloom et al. [20] who observed that by using a probe with a 100 kDa cut-off, the recovery of large proteins decreased significantly *in vitro* after only a few hours. In the present study, the reduction in recovery was only observed *in vivo* and is therefore suggested to be caused by occlusion of the membrane pores by macromolecules in the interstitium. The time course of the decline in recovery after probe implantation is unknown, but the

decrease in FFA concentration at time 110 min. (Fig. 5a) could be due to clogging, since a similar decrease is not seen for glycerol (Fig. 5b). Furthermore, plasma data from the same animals shows a constant FFA concentration throughout the entire time period. Comparison of several sequential treatments might therefore require short sampling periods. However, further studies should reveal the time course for the drop in recovery by testing the probe performance after different lengths of implantation.

The increases in plasma FFA concentrations after treatment with BRL37344 were also lower than the increase for glycerol, although in this case only about three-fold. This could be due to re-esterification of FFA in adipose tissue. Plasma levels are, however, influenced by peripheral utilization of the metabolites and are therefore only an indirect measure of adipose tissue activity and not directly comparable to the dialysate levels. The basal plasma levels for both FFA and glycerol were within the expected range [21,22], as where the responses to the adrenergic stimulation [23].

In conclusion, the present results show that FFA can effectively be sampled by use of microdialysis with good *in vitro* recovery. Lipolytic activity can be measured *in vivo*, although FFA responses are eventually compromised by clogging of the membrane pores limiting extended sampling time periods. Further validation of the technique is therefore necessary before a more adequate FFA response can be obtained *in vivo*. However, the technique holds great potential for future study of local FFA fluxes in order to gain a better understanding of these events together with a more comprehensive dynamic index of adipose tissue metabolism.

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