Assessment of Cortisol Metabolism across Subcutaneous Adipose tissue by Measurements of Arteriovenous Differences: Methode Description

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Introduction
Cortisol is the main glucocorticoid hormone in humans, important in regulation of salt and water balance, stress response and basal metabolism. The adrenal secretes around 5-15 mg cortisol per day. Only a small fraction is excreted as free cortisol in urine, saliva or bile. The rest is cleared by different metabolic pathways before conjugation-and urinary excretion [1]. In the past there were little interest in enzymes regulating cortisol, but the last decade major research programmes have developed finding that the enzymes play a key role in determining local response to the hormone.

The regulation of cortisol access to its receptors involves the hypothalamic-pituitary-adrenal(HPA)-axis and its feedback regulation. Together with signalling and expression of corticosteroid receptors, irreversible metabolism by 5α-and 5β-reductases and reversible metabolism by 11-betahydroxysteroid dehydrogenases (11-BHSD:s) (fig 1), this provides a complex system regulating glucocorticoids in a tissue-specific manner. 11-BHSD type 1 converts cortisone to cortisol and 11-BHSD type 2 catalyze the reaction from cortisol to cortisone (fig 2).

Cushing’s syndrome is caused by an excess of circulating cortisol. Subjects with the metabolic syndrome and patients suffering from Cushing’s syndrome share many symptoms: central obesity with insulin resistance, type 2 diabetes, hypertension and hyperlipidemia. These similarities have led to the hypothesis that cortisol might be a crucial factor in the development of the metabolic syndrome. Several studies have recently shown increased 11-BHSD type 1-activity in subcutaneous fat among obese people [2, 3]. The most significant evidence is the transgenic mice overexpressing 11-BHSD 1 in fat, which develop a complete metabolic syndrome [4, 5]. In addition the 11-BHSD 1 knock-out mice gain less weight and are protected against diabetes despite being fed a high fat diet [6].

With support from prof K Frayn, prof F Karpe (Univ of Oxford) and prof B R Walker (Univ of Edinburgh) we have developed a new method for measurements of 11BHSD-activity across subcutaneous adipose tissue in humans. This is a description of the different techniques that we use in our in vivo studies.

Material and Methods
Adipose tissue is a complex, highly organized tissue with different cell types where the metabolic activity is regulated by a mix of hormones, blood flow and neural input. In order to understand the integration of adipose tissue metabolism, it is necessary to perform in vivo studies. This can be done in a number of ways.

We have infused labelled cortisol [7]. After 3 hours and as a result of 11-BHSD 1 and 2 activity we achieve a steady state across the adipose tissue with different fractions of...
cortisone and cortisol (fig 3). The analyses are made by taking parallel arteriovenous samples from a small vein draining the subcutaneous fat and arterialized blood from a vein draining a warm hand (fig 4). The samples are analysed by gas chromatography combined with mass spectrometry (GCMS). For calculations of adipose tissue blood flow we use injection of the isotope $^{133}$Xe, measuring washout with a portable gamma camera. The techniques are below described in detail.

**Arteriovenous measurements** [8]

The metabolic activity in any tissue is reflected by the differences in the composition of blood sampled from the arterial supply and the venous drainage from the tissue. For example, in a fasting state the concentration of non-esterified fatty acids (NEFA) is higher in the vein draining the fat than in the arterial blood due to net release of NEFA from the adipose tissue. The net release can be quantitatively measured by this calculation:

$$\text{Net release} = \text{Arteriovenous differences} \times \text{blood flow}$$

When choosing the best vein there are some sites where contribution of adipose tissue seems to predominate. Vessels large enough to be identified always carry some drainage from other tissues. In human the most suitable site is the vein draining subcutaneous fat in the anterior abdominal wall [9] (fig 5). It is located 5-7 mm under the skin and can be fairly easy visualized by a fiberoptic lamp. The technique requires a flexible catheter, threaded down a small vein until it is large enough to draw samples. The presence of the catheter allows us to take samples over longer periods, for example before and some hours after interventions (test-meals, lipid infusion etc.). We use a 20 G, 15 cm catheter. To ensure that the blood is collected from the subcutaneous fat, and not from deeper structures, the oxygen saturation is checked to be $\geq 85\%$, adipose tissue has lower extraction of oxygen than other tissues. Although the principle seems easy, the technique needs a lot of training. Forty-five catheterizations in colleagues of mine were required until the technique was working.

In addition to a suitable vein, it is necessary to obtain arterial blood for comparison. The composition of arterial blood is the same all over the body, so access to specific arteries supplying the adipose tissue is not necessary. As an alternative, due to potential problems with arterial cannulation, we use arterialized venous blood [10] from a vein draining a warm hand. Concentrations of most substances are indistinguishable in arterial and arterialized venous blood, except for CO$_2$, lactate and catecholamines. We cannulate the cephalic vein in a retrograde direction with the tip of the cannula at the wrist joint and place the hand in a box (MTI, Huddinge, Sweden) with air warmed to 60${}^\circ$C. The degree of arterialization is depending on the position of the catheter, the state of the subject and the ambient temperature, and it is checked by blood gases to be $>95\%$.

**Adipose tissue blood flow**

There are major differences between adipose tissue blood flow (ATBF) in different depots [11]. The visceral (omental, mesenteral, perirenal, epicardial) depots have the highest flow rates. ATBF is also highly variably in time, responding over a far greater range than skeletal muscle blood flow to a variety of conditions, for example physical and mental stress, food intake, degree of obesity, different hormones and locally produced factors. [12-15]. The adipose tissue blood flow after an overnight fast is typically around 3 ml blood per 100 g
tissue per minute [16]. The theoretical background of using tracer washout techniques is the kinetic model of saturation-desaturation [17], measuring the initial slope of the washout curve, which is proportional to blood flow. Several tracers have been used for blood flow measurements where $^{133}$Xenon is the most widely applied. One of the reasons is its lipid-solubility that allow extended measurements from the same isotope depot. The most commonly used detector is the portable CsI-crystal-detector (Mediscint, Oakfield Instr). The advantages of this system are a small size that allow us to do the studies in an ordinary clinical investigation room, a reasonable price, the possibility to use small doses of isotopes without radiation safety problems [11]. Because of it’s construction, calibration is not needed. The disadvantage of portable detectors is that they may be very sensitive to changes in geometry between the detector and the isotope depot, for example if the subject moves or if you have not put the detector exactly over the injection site. Another problem is that the isotope depot not only moves due to diffusion, but also because of countercurrent exchange between the vein draining the depot and the concomitant artery. This will lead to changed shape of the depot with time and also altered counting efficacy.

1-2 MBq $^{133}$Xenon (gas) is injected subcutaneously paraumbilically. The cannula is kept in situ for at least 1 min after the injection to minimize loss of gas. After an equilibration period of 30-60 min blood flow is monitored by collecting 20 s readings from the portable $\gamma$-camera (Mediscint) placed over the exact injection site. A local depot of 3.7 MBq $^{133}$Xenon will give rise to a whole body radiation less than 0.5 mSv, which is only about 10% of the natural background radiation in most countries. Blood flow is calculated at the exact time of each bloodsampling from a semilog plot of disappearance of counts versus time in 12 min intervalls. ATBF is then calculated according to the equation:

$$ATBF = \text{slope of semilog plot of dissappearance of counts} \times 60000$$  

(60=sec, 100=gram, 10=partition coefficient).

**Deuterium Labeled Cortisol**

Methods used so far determining the activities of 11-BHSD type 1 and 2 in vivo rely on the balance between cortisol and cortisone and do not measure turnover, and cannot distinguish between the two reactions. The use of deuterium labelled cortisol makes it possible to distinguish the type 1 and type 2 activities [7]. This tracer, as prepared by Ulick et al [18] is distinguished from endogenous cortisol by mass difference and is metabolized as shown (in fig 3). On metabolism by dehydrogenation, d4F loses 11$\alpha$-deuterium forming trideuterated cortisone (d3E) and is regenerated by reduction to trideuterated cortisol (d3F).

In our study subjects were given dexametasone the evening before study day in order to suppress the endogenous cortisol production and to avoid potential interference with the morning peak of endogenous cortisol. We start by giving a bolus injection of 3.6 mg of deuterium labelled cortisol (d4F) followed by a constant infusion (1.74 mg/h). After about 3 hours and as a result of type 1 and 2 activity we achieve a steady state across the adipose tissue with different fractions of cortisol and cortisone. Parallell arteriovenous samples are collected at the time for bolus injection, after 1 and 2 hours and every 6th minute between 3 h – 3 h 42 min (steady state). At each timepoint we aim to collect 2 x 5 ml of blood. All samples are immediatly put on ice, centrifuged at 4°C (1000g) for 10 min, whereafter plasma is separated. Analyses are performed using GCMS.

The pharmacokinetic calculations require knowledge of rate of elimination in steady state; these analyses and calculations are done by Dr Ruth Andrew, Univ of Edinburgh.
**Discussion**

Although obese people have normal circulating levels of cortisol, it seems more and more obvious that increased exposure of cortisol in the adipose tissue is an important mediator in development of the metabolic syndrome and its complications. The loss of function or overexpression of 11-BHSD1 in fat may lead to dramatical metabolic consequences, as been demonstrated in transgenic mice. This suggests that tissue specific prreceptor metabolism might be a key regulating step of cortisol exposure. Of major interest is that adipose tissue from obese humans has been shown to have increased expression of 11-BHSD1 [2, 3, 19, 20]. Characterizing the regulation of 11-BHSD1 in human has not been straightforward. Both cortisone and cortisol are metabolized in the liver to tetrahydro-metabolites and earlier studies of cortisone/cortisol metabolites in urine have been contradictory [2, 20-22]. Some studies of obesity [23, 24] have shown subtle alterations in the HPA-axis but these findings have been controversial.

Not surprisingly pharmaceutical companies are working on 11-BHSD1 as an exciting target for treatment of obesity-related complications. The thiazolidinedione (TZD) class of antidiabetic drugs reduce adipocyte 11-BHSD1 mRNA and activity both in vitro and in vivo, suggesting that the visceral fat reducing properties of the TZD:s might be caused by suppression of adipose 11-BHSD1. Currently the 11-BHSD1 inhibitor arylsulfonamidothiazole is in a phase 2 trial for type 2 diabetes. Published data for this substance shows that it is effective in enhancing hepatic insulin sensitivity and lowering blood glucose in diabetic rodents [25, 26].

Another technique that has been used for studying adipose tissue metabolism in vivo is microdialysis, where a small probe is placed in the adipose tissue, allowing hydrophilic substances to diffuse across the membrane making it possible to measure tissue specific concentrations. In theory microdialysis has some advantages compared to the arteriovenous technique, it can be used in different adipose tissue depots and also allows local introduction of effectors, but so far no papers have been published measuring cortisol/cortisone in human adipose tissue by microdialysis.

Regulation of adipose tissue blood flow has been studied extensively. Adrenergic influences by β-mediated vasodilatation and α2-mediated vasoconstriction seems to predominate and may explain increased blood flow during exercise or fasting. Other studies shows that blood flow is modulated by locally produced factors as NEFA [27], nitric oxid, adenosin and prostaglandins. Therefore, different approaches are needed studying adipose tissue function. Most reports on the activity of 11-BHSD:s are based on levels of cortisol and cortisone in blood or the concentrations of their metabolites in the urine. These approaches give limited information of the individual isoenzyme activity, due to the enzymes work in opposition in vivo and these measures merely reflect the balances of their activities. As the isoenzymes possess tissue-specific effects and are altered in different pathologies, measurements of their separate effects are needed. Labelling of cortisol by the stable isotope deuterium makes it possible to distinguish the 11-BHSD type 1 and type 2 activities. It also seems appropriate in an era when radioactive tracers (e.g. 3H or 14C) are ethically unacceptable.

Since the early clinical observations that upper-body obesity was associated with a much greater incidence of diabetes and cardiovascular disease than lower body obesity, much attention has been directed to understand the physiological differences between these sites. Most of this work has been carried out from biopsies, removed at surgery. Due to the complex regulation of adipose tissue metabolism, these in vitro observations must be interpreted with some caution. The general picture is that intra-abdominal adipocytes have the highest metabolic activity, followed by upper-body subcutaneous adipocytes and the lowest responce
in lower-body adipocytes [28]. Except biopsies, specific depots of fat have been studied using microdialysis and depot-specific catheterisations. The vein draining the anterior abdominal wall seems to be the best accessible vessel carrying specific venous drainage from adipose tissue.

Our methode provides a possibility to investigate alterations in 11-BHSD:s within abdominal fat tissue in different populations with a minimum of trauma and discomfort for the participants. It may be possible to use this technique in different metabolic conditions including the effect of various therapeutic interventions.

References


Figures:

Fig 1. Determinants of cortisol access to receptors. CRH=corticotropin releasing hormone. ACTH=adrenocorticotropic hormone.
Fig 2. Cortisol metabolism/clearance. Irreversible metabolism through 5α- and 5β-reductases to THE (tetrahydrocortisone) and THF (tetrahydrocortisol). Reversible metabolism by 11-BHSD:s (11-betahydroxysteroid dehydrogenases).
Fig 3. Metabolism of deuterium-labelled cortisol by the 11BHSD:s.
Fig 4. Assessment of cortisol metabolism across subcutaneous adipose tissue by infusing labelled cortisol, drawing parallel arterio-venous samples and detection of blood flow by Xenon-washout.
Fig 5. Abdominal veins draining the subcutaneous fat. A cannula is inserted in the superficial epigastric vein.