

TECHNIQUES FOR *IN VIVO* ASSESSMENT OF GLUCOSE AND FFA METABOLISM

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ABSTRACT

Obesity and non-insulin dependent diabetes mellitus (NIDDM) are common diseases with multiple pathophysiological disturbances in glucose and lipid metabolism. These defects result from a complex interplay between one or more genetic loci and several environmental factors. Due to this complexity, it is difficult to estimate the relative importance of the individual components, or to assess the efficacy and determine the mode of action of potential new therapeutic agents. The application of various techniques allows to selectively perturb a complex *in vivo* system and to create selective physiological defects in order to determine their potential contribution to obesity and NIDDM. The main focus of this review is on current techniques for *in vivo* assessment of glucose and free fatty acid (FFA) metabolism. We discuss techniques for systemic measurement including clamp methods, isotope tracer methods, and indirect calorimetry. The outlined regional measurement techniques are: the arteriovenous differences, the microdialysis and the microperfusion for sampling interstitial fluid from muscle and adipose tissue, and the nuclear magnetic resonance (NMR) spectroscopy and the positron emission tomography (PET). Mathematical modeling approaches, like the minimal models for glucose utilization and deconvolution for reconstructing endogenous fluxes (hepatic glucose production and insulin secretion) are highlighted. The discussion of animal models includes the most common rodent models and transgenic approaches. Finally, the frequently used analytical techniques are briefly summarized. In summary, assessment of glucose and FFA metabolism is a scientific challenge, but with modern techniques, it is possible to further explore the cellular and molecular events involved in the regulation of these metabolic processes and develop therapeutic agents for the treatment of obesity and NIDDM.

KEY WORDS: non-insulin dependent diabetes mellitus, insulin resistance, clamp techniques, isotope tracer, microperfusion, mathematical modeling, animal models, analytical techniques.

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LIST OF SYMBOLS

g	Plasma glucose concentration, $\text{mmol}\cdot\text{l}^{-1}$
g_b	Basal plasma glucose concentration, $\text{mmol}\cdot\text{l}^{-1}$
i	Plasma insulin concentration, $\mu\text{U}\cdot\text{ml}^{-1}$
i_b	Basal plasma insulin concentration, $\mu\text{U}\cdot\text{ml}^{-1}$
p_1	Constant parameter, min^{-1}
p_2	Constant parameter, $\text{ml}\cdot\mu\text{U}^{-1}\cdot\text{min}^{-2}$
p_3	Constant parameter, min^{-1}
S_I	Insulin sensitivity, $\text{min}^{-1}\cdot\text{ml}\cdot\mu\text{U}^{-1}$
S_G	Glucose effectiveness, min^{-1}
$h(t)$	Impulse function
$R_a(t)$	Flux, $\text{mol}\cdot\text{min}^{-1}$
$Q(t)$	Mass of a substance, mol
Q_N	Unlabeled tracee mass, mol
q_N	Labeled tracee mass, mol
Q_I	Unlabeled tracer mass, mol
q_I	Labeled tracer mass, mol
q_I^*	Radiolabeled tracer mass, mol

ABBREVIATIONS

ACC.....	Acetyl-CoA carboxylase
ATP.....	Adenosine triphosphate
cDNA.....	Complementary deoxyribonucleic acid
CE.....	Capillary electrophoresis
CL.....	Citrate lyase
CPT-1.....	Carnitine palmitoyltransferase 1
DNA.....	Deoxyribonucleic acid
ELISA.....	Enzyme-linked immunoassay
FFA.....	Free fatty acid
G6P.....	Glucose-6-phosphate
GABA.....	γ -aminobutyric acid
GC-MS.....	Gas chromatography-mass spectrometry
GLC.....	Gas-liquid chromatography
GLUT4.....	Glucose transporter protein 4
HGP.....	Hepatic glucose production
HPLC.....	High-performance liquid chromatography
HPCE.....	High-performance capillary electrophoresis
HSL.....	Hormone sensitive lipase
IDDM.....	Insulin-dependent diabetes mellitus
IGF-1.....	Insulin-like growth factor 1
IRMS.....	Isotope ratio mass spectrometry
IRS-1.....	insulin receptor substrate 1
IRS-2.....	insulin receptor substrate 2
IVGTT.....	Intravenous glucose tolerance test
LC-CoA.....	Long-chain acyl-CoA
LPL.....	Lipoprotein lipase
MIDA.....	Mass isotopomer distribution analysis
mRNA.....	Messenger ribonucleic acid
MS.....	Mass spectrometry
NIDDM.....	Non insulin-dependent diabetes mellitus
NMR.....	Nuclear magnetic resonance
OGTT.....	Oral glucose tolerance test

PAGE.....	Polyacrylamide gel electrophoresis
PCR.....	Polymerase chain reaction
PET	Positron emission tomography
RIA	Radioimmunoassay
RT-PCR.....	Reverse transcription polymerase chain reaction
TCA.....	Tricarboxylic acid
TG.....	Triglyceride
TNF- α	Tumor necrosis factor alpha
VLDL.....	Very low density lipoprotein

1. INTRODUCTION

Non-insulin-dependent diabetes mellitus (NIDDM) is a common disease in Western industrialized societies reaching epidemic proportions [1]. NIDDM is the third most prevalent disease affecting 5% of the population of Europe and North America and about 100 million worldwide. NIDDM is responsible for a significant amount of morbidity and mortality, primarily through associated dyslipidemias, atherosclerosis, hypertension, cardiovascular disorders, and renal dysfunction [2]. The significance of this problem was recognized very early and an increasing number of studies have been conducted since the landmark discovery by Minkowski in 1889, finding that the pancreas produced some entity that controls blood sugar level. Despite enormous investigative effort, a clear understanding of the pathogenesis of this disease and of the temporal relationship between the basic pathophysiological mechanisms has not yet emerged.

Approximately 85% of the patients with NIDDM are obese and it has been known for decades that obesity is accompanied by profound disturbances in glucose metabolism, especially in the action of insulin, the main hormone regulating glucose metabolism. The connection between obesity and NIDDM is poorly understood, and the molecular mechanisms that are involved are still not known. Obesity and NIDDM have one major common feature: they are always associated with insulin resistance, the earliest detectable metabolic defect [3]. Thus, elucidating the insulin-glucose axis as a central component of fuel homeostasis has been the main objective in many research studies.

It has recently been suggested that NIDDM may have more to do with abnormalities in fat than glucose metabolism [4]. Indeed, another major feature characterizing patients with NIDDM is elevated circulating levels of free fatty acids (FFA). NIDDM may therefore be considered a disease of glucose tolerance as well as a lipid disorder. Thirty years ago Randle [5] demonstrated that the increased availability of FFA decreased glucose oxidation and suggested that there is a reciprocal relationship between the utilization of glucose and lipids for

energy production: the so-called glucose-fatty acid cycle or Randle cycle. The Randle cycle has gained renewed attention during the last few years [6] and several groups have reexamined the glucose-fatty acid interactions *in vivo* using various techniques.

However, the importance of the cycle in the pathogenesis of insulin resistance and the effect of FFA is still controversial. Additional studies are required to further investigate the interactions between glucose and FFA metabolism in humans and to characterize the metabolic processes in distinct tissues. In order to investigate the interactions between FFA and glucose metabolism, measurements of uptake and production of these metabolites and their substrates in various tissues are needed.

In recent decades a variety of analytical techniques with high specificity and sensitivity, methods for clinical investigations, and animal models were developed which can be applied for studying glucose and lipid metabolism. Many of these *in vivo* and *in vitro* techniques are not limited to glucose/lipid metabolic studies. It is well known that insulin, the major metabolic factor regulating glucose metabolism is also a growth factor and that insulin signal transduction pathway is closely related to the cell proliferation and differentiation signaling pathways. Alterations of signaling pathways can result in dysregulation of cell growth, and such mutations are associated with some human cancers. Therefore, oncology is a field which has been greatly affected by advances in the understanding of signal transduction. Due to the higher specificity, signal transduction is an attractive target for the development of new antiproliferative drugs. Such a specific approach may avoid the toxicities associated with standard cytotoxic agents. Thus, many of the techniques presented in this review might be also helpful for this nontraditional approach to drug development.

The main focus of this review is on the current techniques for *in vivo* assessment of glucose and lipid metabolism. State-of-the-art, advantages and disadvantages of the various methods as well as their limitations and applicability are highlighted

with respect to current trends. After a brief description of the physiology and pathophysiology of the glucose and FFA metabolism and their interaction, we will discuss techniques for systemic and regional measurement. Clamp techniques, isotope tracer techniques and indirect calorimetry are widely used for systemic measurement, and are therefore outlined herein. Microdialysis and microperfusion are recently developed techniques for assessment of regional metabolism that enable sampling of interstitial fluid from muscle and adipose tissue in both humans and animals. Their potential application for sampling small molecules and macromolecules as well as various calibration methods are discussed. Furthermore, the chapter for regional measurements includes also nuclear magnetic resonance (NMR) spectroscopy and positron emission tomography (PET). Mathematical techniques like the minimal models for glucose utilization and deconvolution for reconstructing endogenous fluxes (insulin secretion, hepatic glucose production) are now also established methods. The methods which elucidated many mechanisms in molecular physiology and increased our understanding in the past few years are animal models, including the transgenic and gene knockout models. Finally, commonly used analytical techniques are briefly summarized.

Given the multitude, the complexity, and the interaction with other mechanisms, this review can not be and is not intended to be an exhaustive primer for techniques for investigation of both glucose and FFA metabolism. Moreover, the developments in this field are highly dynamic and cover many fields in life sciences like biophysics, biochemistry, molecular and cell physiology and biology, genetics, pharmacology, or clinical research. Therefore, this review highlights certain techniques which are either widely used, represent 'golden' standard, or are highly promising for future research and therapy. Furthermore, in order to keep the number of citations small, wherever possible and appropriate, recent review papers were referred.

2. GLUCOSE AND FFA METABOLISM

Animals and humans use FFA and glucose as fuels for energy production under different conditions in different tissues. During fasting, skeletal muscle, heart and liver preferentially use FFA as energy source whereas glucose is the major fuel used by the brain. After ingestion of carbohydrates, the utilization of FFA is suppressed and the uptake and utilization of glucose are enhanced. Glucose and FFA are also used for energy storage. However, storage of FFA in the form of triglycerides (TG) is more efficient and quantitatively more important than storage of glucose as glycogen. Clearly, to maximize energy production and storage, these fuels are subject to an integrated and complex regulation. The major pathways of glucose and FFA metabolism and their regulation have been previously described in detail [7]. In the following, the glucose and FFA metabolism and their interaction are briefly summarized and specific pathological defects with respect to obesity or NIDDM are pointed out.

2.1 GLUCOSE METABOLISM

Transport for glucose into the cells can be dependent on the presence of insulin, like in adipose, heart and muscle cells, or can be mediated in an insulin-independent manner, like in the brain and red blood cells. Among the seven genes that form the superfamily of glucose transporters, the insulin sensitive isoform has been identified, cloned, and named glucose transport protein 4 (GLUT4) [8]. Studies performed in obese subjects have demonstrated that glucose transport displays defects in the expression of the GLUT4 gene, as shown by the reduced levels of GLUT4 mRNA [9]. However, muscle cells are not depleted of GLUT4 mRNA [10], thus indicating that the decreased glucose transport must be due to defects located in the translocation and/or activation processes of GLUT4.

Glucose is used by all cells of the body, by extracting part of the chemical energy inherent in the glucose molecule. After penetrating the plasma membrane, glucose

is metabolized differently in various cells (Fig. 1). The first intermediate is glucose-6-phosphate (G6P), which is result of glucose phosphorylation by hexokinase. G6P can be converted to glycogen (glycogenesis) in muscle and heart tissue cells, in adipose cells, and in liver parenchymal cells. The other pathway for G6P is utilization by glycolysis to give pyruvate and lactate. It is noteworthy that oxygen is not necessary for glycolysis, and the presence of oxygen can indirectly suppress glycolysis. Due to the lack of mitochondria, the end product of glycolysis in red blood cells is lactate, which is released from the cells back into the blood plasma. In the brain, muscle and liver cells, pyruvate can be utilized by the pyruvate dehydrogenase complex and the tricarboxylic acid (TCA) cycle within the mitochondria to provide energy. In the adipose cells, pyruvate generated by glycolysis can be oxidized by the pyruvate dehydrogenase complex to give acetyl CoA, which is used primarily for *de novo* fatty acid synthesis.

Blood glucose levels must be maintained to support metabolism of those tissues that use glucose as their primary substrate and therefore, the capacity to synthesize glucose is crucial. One process of glucose formation is glycogenolysis, i.e. glucose or G6P formation from glycogen. Glucose can be synthesized or formed from a large variety of non-carbohydrate substrates (gluconeogenesis) including various amino acids, lactate, pyruvate and glycerol. Glucose can be also synthesized from galactose and fructose.

There are two important cycles in gluconeogenesis that provide mechanisms for continuously supplying glucose to tissues: the Cori cycle and the alanine cycle. The Cori cycle consists of gluconeogenesis in the liver followed by transport of glucose to a peripheral tissue, glycolysis to lactate and lactate transport to the liver. The major difference between the Cori and the alanine cycle is the type of recycled three-carbon intermediates: in the alanine cycle glucose is converted to pyruvate and the carbon returning to the liver is alanine. The cycles are functional between the liver and tissues that do not completely oxidize glucose to CO₂ and H₂O.

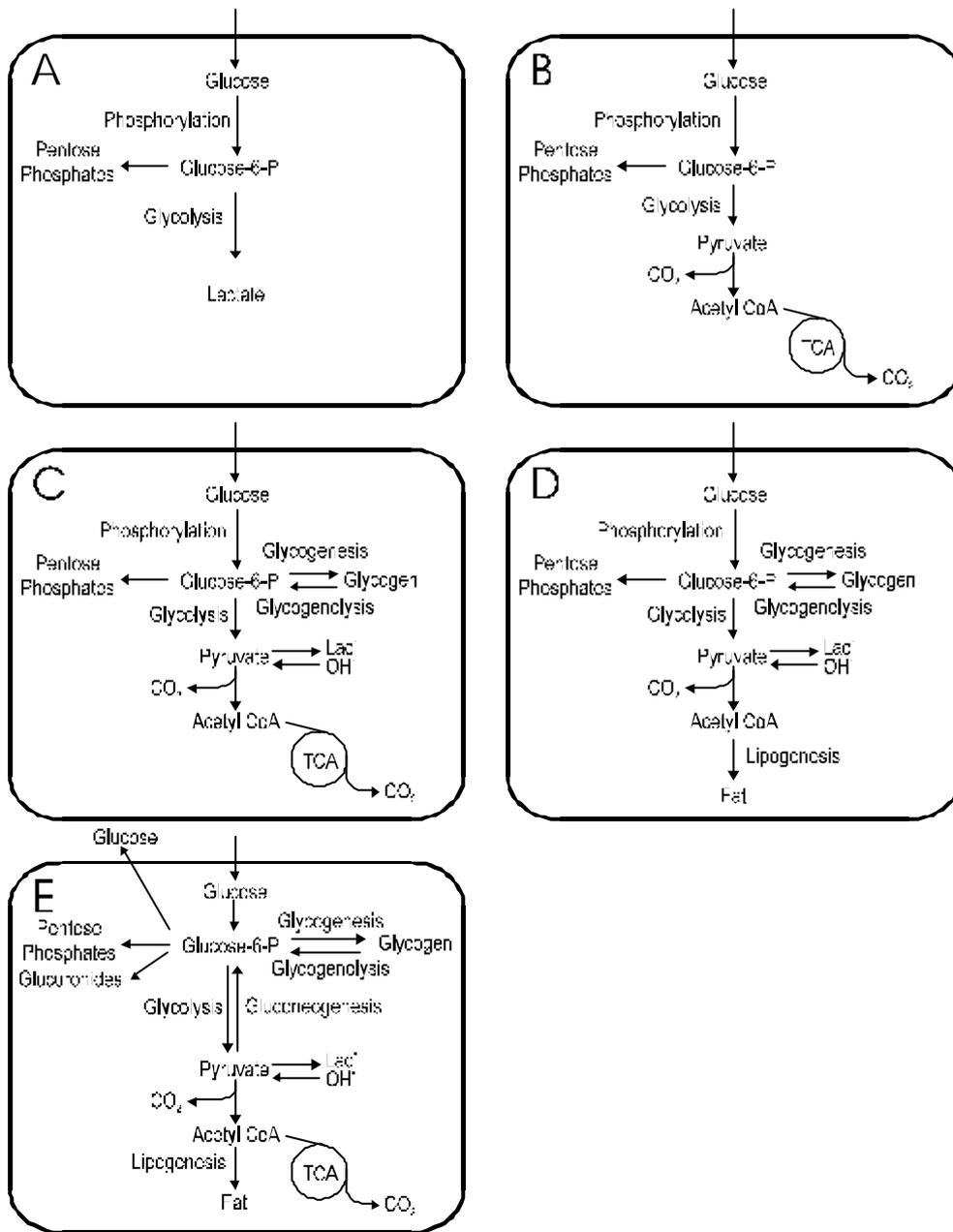


Fig. 1: Major pathways in which glucose is metabolized within cells of selected tissues of the body. A: Red blood cells. B: Brain tissue cells. C: Muscle and heart tissue cells. D: Adipose tissue cells. E: Liver parenchymal cells. Glucose 6-P: glucose 6-phosphate. TCA: tricarboxylic acid.

2.2 FFA METABOLISM

One of the most prominent properties of FFAs and TGs is their hydrophobic nature which makes them efficient compounds for storing energy. TGs can be stored as pure lipid without associated water, whereas glycogen is hydrophilic and binds about twice its weight of water when stored in tissues. Moreover, an average person stores about 100g of carbohydrate as liver glycogen and 250g as muscle glycogen, which is about 1400 kcal of available energy. This amount is barely enough to sustain body functions for 24 h of fasting. However, the normal complement of fat stores will provide sufficient energy to allow several weeks of survival.

A great proportion of the FFAs utilized by humans is supplied by their diet. Besides dietary supply, an alternate source is their biosynthesis from small-molecule intermediates. After synthesis or dietary supply the FFAs are transported in the blood to various tissues. The human body uses three types of substances as vehicles to transport lipid-based energy: 1) chylomicrons in which TGs are carried in protein-coated lipid droplets. This represents a transport of dietary fatty acids throughout the body from the intestine after absorption. 2) FFAs bound to serum albumin, which is a transport of energy released from the storage in adipose tissue to the rest of the body and 3) ketone bodies, the most soluble form of lipid-based energy, i.e. acetoacetate and β -hydroxybutyrate. In this case lipid-based energy processed by or synthesized in the liver and distributed either to the adipose tissue for storage or to other tissues for utilization is transported in ketone bodies and other plasma lipoproteins like the very low density lipoproteins (VLDL).

The energy in fatty acids stored or circulated as TG is not directly available and must be enzymatically hydrolyzed to release FFAs and glycerol. Two types of lipases are involved in the hydrolysis: lipoprotein lipase (LPL) located on the surface of the endothelial cells of capillaries which hydrolyze TG in the plasma lipoproteins and the hormone-sensitive lipase (HSL) which hydrolyze TG in

adipose tissue. The available FFAs are taken up by the cells and oxidized inside the mitochondria (Fig. 2). The first step in the oxidation is the activation of a fatty acid to a fatty acyl CoA. However, the mitochondrial membrane is impermeable to CoA and a shuttle system is necessary to transfer the acyl group to carnitine in a reaction catalyzed by carnitine palmitoyltransferase 1 (CPT-1). The acyl carnitine exchanges across the membrane with free carnitine and is transferred back to CoA.

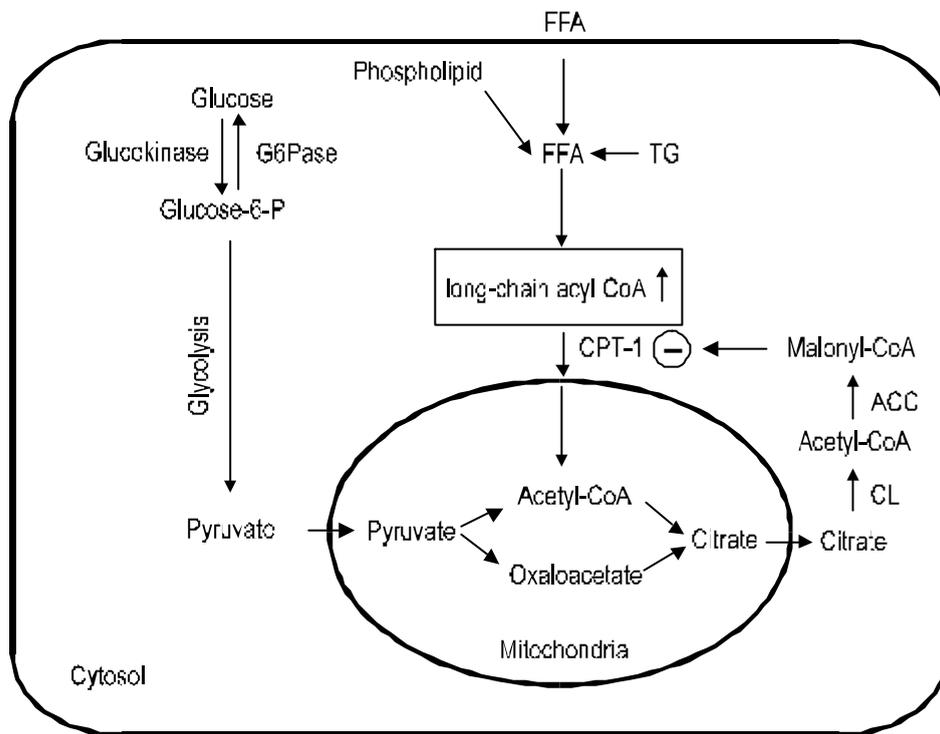


Fig. 2: Cytosolic long-chain acyl CoA (LC-CoA) regulation. The entry of LC-CoA into the mitochondria is controlled by malonyl-CoA which regulates CPT-1. ACC: acetyl-CoA carboxylase. CL: citrate lyase. G6Pase: glucose-6-phosphatase. TG: triglyceride.

2.3 Interactions between glucose and FFA Metabolism

The first theory to explain the effect of increased FFA oxidation on the uptake and oxidation of glucose was based upon experiments performed with perfused rat heart and diaphragm [5]. Randle *et al.* [5] found that the addition of fatty acids and ketone bodies to the perfusion medium reduced glucose uptake in hearts of normal rats to levels comparable to those seen in diabetic or starved rats. According to this cycle, an excess of circulating FFAs, originating from an increased fat mass, could induce a state of insulin resistance in skeletal muscle via a substrate competition for oxidation. Furthermore, an increase in FFA oxidation could enhance the rate of gluconeogenesis [11]. Thus, elevated plasma FFA concentration observed in most obese subjects produce peripheral and hepatic insulin resistance. Recent studies identified two mechanism how obesity produces insulin resistance [12]: 1) by an FFA-related inhibition of glucose transport or phosphorylation and 2) a decrease in muscle glycogen synthase activity. In normal subjects this resistance is compensated by FFA-induced potentiation of glucose stimulated insulin secretion [13]. It was proposed that, in the development of NIDDM, FFAs fail to stimulate insulin secretion, resulting in hepatic overproduction and peripheral under-utilization of glucose [12].

2.4 Leptin, TNF- α and insulin resistance

Recent discoveries suggest that besides FFA there are two additional products of the fat cell that might have a key role in the insulin resistance in obesity and NIDDM, namely the leptin [14] and tumor necrosis factor-alpha (TNF- α) [15]. The positional cloning of the mouse obese (*ob*) gene in the Friedman's laboratory initiated a large number of research studies on obesity. It is now clear that leptin, a 16-kDa protein and product of the *ob* gene acts as a hormone at the level of hypothalamus to restrain food intake and increase energy expenditure [16]. Leptin plasma concentrations are increased in human obesity, although this increase is not accompanied by increased energy expenditure or decreased appetite. Thus,

human obesity is a leptin-resistant disorder. Whether leptin-resistance is due to a defect in leptin receptor or postreceptor signal transduction and whether it involves other mechanisms or has direct metabolic effects is presently unknown and needs to be further assessed.

TNF- α is a cytokine that is prevalently produced by macrophages and plays a role in inflammatory and immune processes [15]. It was also shown that fat tissue produces TNF- α and that its expression is elevated in rodent models of human obesity. TNF- α inhibits LPL activity in adipose tissue and intracellular signaling [17]. Circulating levels of TNF- α are very low in insulin resistant conditions but the TNF- α expression is increased in adipose tissue. Thus, in the model for the role of TNF- α in the obesity-linked insulin resistance an autocrine/paracrine effect was proposed [17]. However, due to technical difficulties, *in vivo* assessment of TNF- α in muscle and adipose tissue was not possible so far.

Above and beyond providing a clear explanation of obesity-linked insulin resistance, FFAs, leptin and TNF- α might provide new therapeutic options. For example, nicotinic acid analogs inhibit adipocyte lipolysis and decrease circulating FFA levels. This 'reversal' of the glucose-FFA cycle, induced by decreased FFA availability improves glucose metabolism. It is also anticipated that an antagonist of TNF- α (anti-TNF- α antibody) may attenuate or cure insulin resistance. However, further studies will be necessary to assess the role of the TNF- α and leptin and the therapeutic interventions based on this adipocyte products in human obesity and NIDDM.

3. SYSTEMIC MEASUREMENTS

From the historical point of view, systemic measurements were the first methods for investigating glucose and FFA metabolism *in vivo*. Measurements of circulating levels of various metabolites and hormones are performed routinely and are the most convenient methods for large scale population studies. Another routinely used method is the oral glucose challenge: the oral glucose tolerance test (OGTT). Certain amount of glucose (75-100g) is given orally and the blood glucose and insulin levels are measured for the following 2-3 hours. The area under the curve gives a rough estimate about the patient's glucose tolerance. However, simple measurements of the circulating glucose levels or OGTT are not sufficient to estimate distinct processes like disposal and production of glucose in specific tissues. Therefore, more sophisticated techniques were developed and established in the last years. These methods are: the clamp techniques, infusions of radiolabeled or stable-labeled isotope tracers, indirect calorimetry, and a combination of these techniques.

Independent of the technique used, the choice of the infusion and sampling site is crucial and may have a significant effect on the calculated metabolic fluxes. The usual assumption is that the mixing of infusate is complete and instantaneous. This holds true only in the case in which the mixing occurs rapidly relative to the washout kinetics of the tracer. Due to the accessibility, usually the venous site is used for drawing blood samples. In cases where infusion and sampling are necessary there are two choices: arterial infusion and venous sampling (A-V mode) or venous infusion and arterial sampling (V-A mode) (Fig. 3). For studies of whole body glucose metabolism the latter is more appropriate due to problems of inadequate mixing of an infusion mixture in the artery and a potentially unequal distribution of the tracer [18]. In the case of glucose, the A-V mode would result in overestimation of the hepatic glucose production (see Chapter 3.2 for the calculation of hepatic glucose production) because sampling occurs before the mixing of the tracer and hence, underestimation of the tracer enrichment.

Conveniently, instead of arterial sampling, sampling of arterialized venous plasma is performed: a hand vein is cannulated retrogradely and is kept in a thermoregulated ($55\text{-}60^{\circ}\text{C}$) box. Measurement of capillary, arterial, arterialized venous and venous plasma during hyperinsulinemic euglycaemia has shown that there are only small differences in glucose between the arterial and arterialized venous plasma [19].

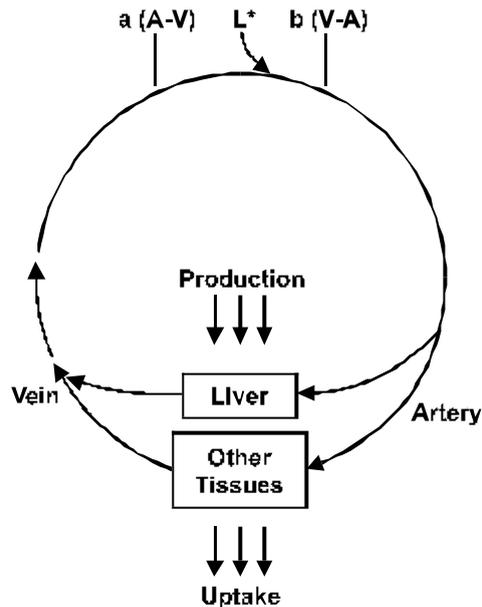


Fig. 3: Model for the choice of the sampling and infusion site [18]. The tracer is infused at point L^* . Sampling at point **a** refers to the A-V mode (arterial infusion and venous blood drawing) and sampling at point **b** refers to the V-A mode (venous infusion and arterial blood drawing).

3.1 Clamp techniques

The 'gold standard' for measurement of the insulin-stimulated peripheral uptake of glucose, or insulin sensitivity is the hyperinsulinemic euglycemic clamp technique [20]. During the clamp, insulin concentration is fixed by a primed continuous infusion of insulin at a certain level (Fig. 4). This can be either hypoinsulinemic (with somatostatin infusion to suppress insulin secretion) or hyperinsulinemic at low or high levels. The plasma glucose concentration is clamped at a desired level by a variable infusion of glucose. Again, the desired level can be hypoglycaemic, euglycaemic, or hyperglycaemic. The rate of glucose

infusion necessary to maintain constant glucose concentrations is adjusted according to frequent measurements (3-10 min) of plasma glucose. During the steady state of the euglycemic hyperinsulinemic clamp, the hepatic glucose production is completely suppressed by insulin (at high insulin levels) and the amount of glucose infused equals the amount of glucose taken up by the peripheral tissues.

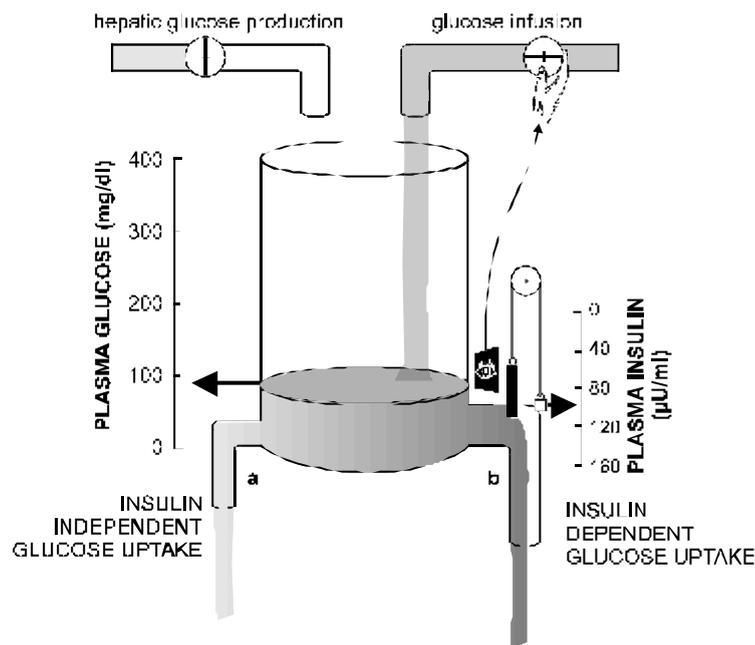


Fig. 4: Schematic representation of the euglycemic hyperinsulinemic clamp technique (adapted from [25]). Insulin is infused at constant rate to achieve elevated plasma insulin concentrations (hyperinsulinemic). Glucose is infused at a variable rate to maintain constant plasma glucose concentration at normal levels (euglycemic). At high insulin levels, there is no hepatic glucose production and the glucose infusion rate equals the peripheral glucose uptake. The glucose uptake is insulin dependent (**b**) and insulin independent (**a**).

The glucose clamp technique was modified in order to be able to study counterregulatory responses [21]. By infusion of different agents to block endogenous secretion and replacement of specific hormones during hypoglycemia, it is possible to study pathological changes of epinephrine, norepinephrine, glucagon, cortisol, somatostatin, and human growth hormone. This technique is useful to assess the contribution of individual hormones during

hypoglycemia by creating a deficiency of a particular hormone. Additional advantage of this pancreatic clamp method is that the circadian variations of the hormone concentrations are eliminated.

Another modification is the simultaneous infusion of TG during euglycemic hyperinsulinemic clamps to elevate the plasma FFA concentrations [22]. High levels of plasma FFA were produced by the infusion of TG and heparin, which stimulates LPL and hence, hydrolysis of TG. Using this technique it was demonstrated that the inhibition of insulin-stimulated glucose uptake becomes statistically significant approximately 3.5 h after the start of the lipid infusion [22].

3.2 Tracer techniques

Systemic appearance rates of metabolites like glucose or FFA are either not accessible to measurement or are very difficult to access. Isotope tracer methods, either with radioactive or stable-label tracers are superior and have long been used to measure systemic rates [18]. An ideal tracer is chemically identical to the substance of interest (tracee), but different in some characteristics that enables its detection. Tracers can be radioactive, like ^{14}C and ^3H or stable isotopes like ^2H and ^{13}C . Stable-labeled tracers have gained increased interest due to the noninvasivity and higher analytical precision which can be achieved using mass spectrometry gas-chromatography instruments (GS-MS). However, there is still a role for radioactive tracers, especially in animal models. Many of the principles of kinetic modeling in the context of radiolabeled isotopes apply also to stable-labeled isotopes. The major difference between the radiolabeled and the stable-labeled tracers is given in Fig. 5 [23]. Due to the natural abundance stable isotopes are present in the body and the tracer may not have negligible mass. Also the analytical procedure used for the quantitation of the sample is fundamentally different. However, a kinetic formalism for the analysis of stable isotope tracer

data and its link with the radioactive kinetic formalism was established recently [23].

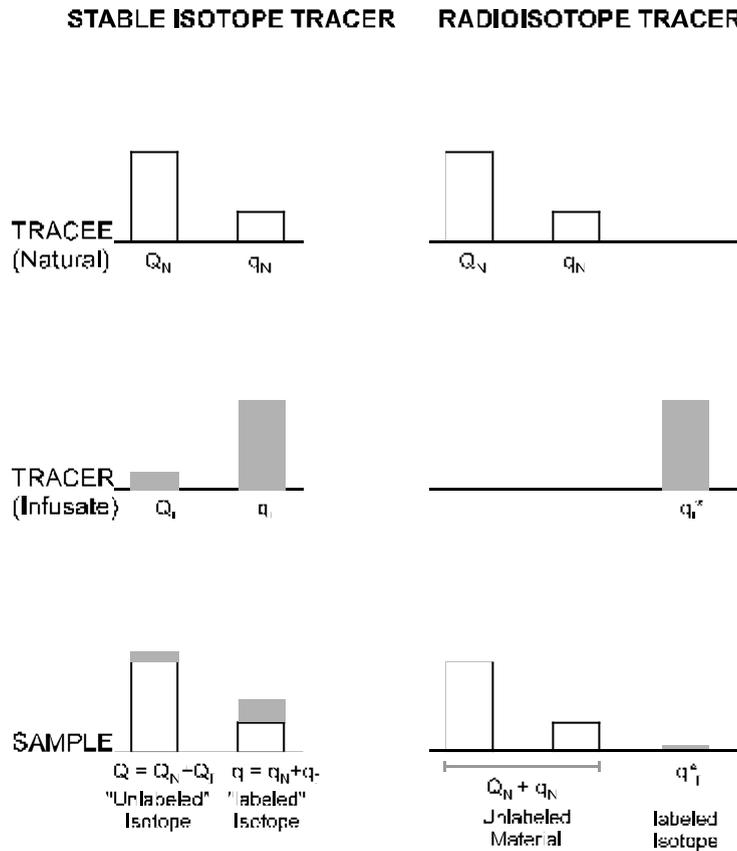


Fig. 5: Radiolabeled and stable-labeled tracer and their relative contribution to tracee (endogenous material, subscript N), tracer (infused material, subscript I), and sample (adapted from [23]). Q: unlabelled species. q: labeled species. q^* radiolabeled species. In the case of stable isotopes there is a certain amount of naturally occurring stable isotopes in the tracee.

The use of glucose tracer in combination with a euglycemic hyperinsuliemic clamp with submaximal insulin stimulation enables the separation of endogenous and exogenous (i.e. infusate) glucose and hence, the calculation of hepatic glucose production (HGP) and glucose disposal. Most commonly, the tracer is infused at a primed, constant rate during the experiment, allowing a 2-3 hour equilibration period before the beginning of insulin infusion. However, most likely due to an expansion of the glucose space [24-25], the estimates of HGP are erroneously negative. This problem is avoided if the specific activity (or enrichment for stable isotopes) of glucose in plasma is kept constant, the so-called "hot glucose" method

[26]. This is achieved by administering labeled glucose together with the infused variable glucose infusion. The specific activity of the infused glucose is estimated based upon an assumption of the rate of basal hepatic production [26].

Gluconeogenesis can be determined indirectly by NMR spectroscopy (see chapter 4.4) or directly by using isotopes. The NMR spectroscopy is usually combined with infusion of labeled glucose for the determination of HGP. From the NMR spectra one can calculate the glycogenolysis and using both, HGP and glycogenolysis, the gluconeogenesis. Recently, a method based on $^2\text{H}_2\text{O}$ incorporation was developed by Landau *et al.* [27]. After oral administration of $^2\text{H}_2\text{O}$, which resulted in low body water enrichment (0.5%), the gluconeogenesis was determined by comparing the ^2H enrichment into C6-glucose to the ^2H enrichment into C2-glucose [27] using sensitive analytical procedure. Another technique uses labeled glycerol and mass isotopomer distribution analysis (MIDA) [28] to estimate gluconeogenesis. MIDA is a technique for measuring synthesis or turnover of polymers, i.e. products containing two or more repeating units (monomers). First, the pool of monomers is enriched by constant infusion and then, by measuring the distribution of the label in the polymer and using mathematics of combinatorial probabilities, the monomer enrichment is deduced [28]. However, using these techniques, NMR spectroscopy, $^2\text{H}_2\text{O}$ incorporation or MIDA, inconsistencies in the literature have occurred and further studies will be necessary to clarify the contradictions.

To measure the rate of adipose tissue lipolysis, measurements of systemic appearance rates of FFA and glycerol by isotope tracer methods can be performed. Since there is little glycerol kinase activity in adipose tissue and glycerol produced from lipolysis is released into the circulation, glycerol appearance rate reflects lipolysis [29]. When using FFA tracer, the turnover rate of FFA may not accurately reflect lipolysis due to FFA reesterification or storage in the intracellular pool. Furthermore, labeled FFAs have to be bound to albumin to be infused. Since the commercially available human albumin is not genetically

produced but collected from several donors, concerns arised, mostly from recent reports on prion diseases infusing albumin in human studies.

The rate of fatty acid synthesis or *de novo* lipogenesis can be quantitated by measuring the incorporation of an isotopic precursor into the FFA pool. Work with purified fatty acid synthase showed that the hydrogens in palmitic fatty acid are derived from NADPH, acetyl CoA and water. Thus, by labeling the pools of one of these precursors and measuring the product (palmitate), the *de novo* lipogenesis can be determined. Acetate, either radiolabeled or stable-labeled, is the commonly used substrate for the quantitation of fatty acid synthesis [28].

Cellular water is a relevant precursor to hepatic *de novo* lipogenesis since the water hydrogens are incorporated into fatty acids. Hence, using labeled water enables the calculation of the synthesis of fatty acids. However, due to the very high doses required for this procedure using $^3\text{H}_2\text{O}$, the procedure was not used in humans. Recently, a method using stable labeled water ($^2\text{H}_2\text{O}$) and isotope ratio/mass spectrometry (IR-MS) for the estimation of hepatic *de novo* lipogenesis in humans was developed [30]. Since the deuterium enrichment in plasma TG and cholesterol was low, a tedious and time-consuming procedure for extensive purification of TG and cholesterol, combustion of the purified compounds and water reduction was required [30]. More recently, using a higher plasma enrichment but still well-tolerated and without side effects, a simpler GS-MS method was developed [31].

3.3 Indirect calorimetry

Indirect calorimetry is a relatively simple method of measuring oxygen consumption, carbon dioxide production, and urinary nitrogen excretion, and is widely used to assess energy expenditure and net oxidation rates [32]. In a resting subject the energy expenditure corresponds to heat production, since the total amount of energy used within the body is transformed into heat. The term *indirect*

means that the heat released by chemical processes is indirectly calculated from the rate of oxygen consumption $\dot{V}O_2$. Since almost all processes requiring energy need oxygen consumption, there is a close relationship between energy expenditure and $\dot{V}O_2$. Anaerobic glycolysis provides adenosine triphosphate (ATP) without oxygen consumption but it represents a small percentage of ATP production under normal circumstances.

The procedure that has become the method of choice to determine energy expenditure measures oxygen consumption with an open canopy circuit which is constantly ventilated with fresh air. Fractions of oxygen and carbon dioxide are monitored at the inlet and the outlet of the canopy by gas analyzers. The total volume of air flow through the system is measured and consequently, the rate of total oxygen consumption ($\dot{V}O_2$), the rate of carbon dioxide production ($\dot{V}CO_2$), and the respiratory quotient ($\dot{V}CO_2/\dot{V}O_2$) are calculated from these values [33]. Additionally, urinary nitrogen excretion (N) is simultaneously measured. From these measurements, the rate of oxidation of glucose, lipid, and protein can be calculated [32, 33] as:

$$\text{Glucose oxidation rate (g/min)} = 4.62\dot{V}CO_2 - 3.28\dot{V}O_2 - 3.64N$$

$$\text{Lipid oxidation rate (g/min)} = 1.706\dot{V}O_2 - 1.706\dot{V}CO_2 - 1.76N$$

$$\text{Protein oxidation rate (g/min)} = 6.25N$$

These equations are derived from the values of consumed O_2 and produced CO_2 per gram of substrate (glucose, lipid and protein) and taking account that nitrogen is about 16% of protein by weight [32].

Measurements of indirect calorimetry are usually carried out during periods of 30 min in order to compensate for the transient effects of hyperventilation, i.e. increased $\dot{V}CO_2$, by periods of hypoventilation. Since the body has negligible oxygen stores, the $\dot{V}O_2$ immediately corresponds to the actual oxygen consumption in the tissue. Note, that in the postabsorptive state the carbohydrate

energy source is glycogen and not glucose. Therefore, the appropriate coefficients for the glucose oxidation rate have to be multiplied by the molecular weight of a glucosyl residue divided by the molecular weight of glucose (162/180). One should have in mind that the estimation of glucose oxidation provides estimates of net glucose oxidation which includes: 1) oxidation of glucose from endogenous glycogen stores or exogenous carbohydrates, 2) glucose conversion to lipids with concomitant lipid oxidation, and 3) glucose conversion to amino acids with concomitant oxidation of amino acids.

Since indirect calorimetry is a noninvasive technique, it can be advantageously combined with other experimental techniques to provide a powerful tool to examine glucose and FFA metabolism. This technique is usually combined with clamp techniques. During hyperinsulinemic euglycemic clamps or in postprandial conditions, hyperinsulinemia inhibits net protein and net lipid oxidation rates. Hence, the true glucose oxidation rate is similar to the net carbohydrate oxidation rate. Additionally, tracer techniques can be used with indirect calorimetry to measure the turnover rate of various substrates. In summary, by combining the techniques for systemic measurement: indirect calorimetry, insulin clamp and isotope tracer techniques, the contribution of changes in circulating hormone levels, substrates and metabolites can be assessed in a variety of pathophysiological states.

4. REGIONAL MEASUREMENTS

Systemic measurements allow direct quantitation of whole-body metabolic rates and are not dependent on blood flow. The studies at the whole-body level provide an integrated picture of metabolic activity without distinguishing the differences in the regulation and activity of metabolism in different regions. If the question being addressed is the contribution of various tissues in total metabolism or local variations and/or regulation, whole-body methodologies are not appropriate. Regional measurement can be performed using the arteriovenous differences technique, microdialysis, microperfusion, and NMR and PET techniques.

4.1 ARTERIOVENOUS DIFFERENCES

The arteriovenous differences have long been applied to forearm muscle [34], liver [35], and have more recently been used for subcutaneous adipose tissue [36]. The method is based upon measurement of metabolite concentrations in arterial and venous blood in specific tissues. If determination of arteriovenous differences is combined with measurements of the rate of blood flow through the tissue of interest, it is possible to measure uptake and output of a given metabolite quantitatively. This technique is restricted to humans and large animals and it is relatively difficult and time-consuming. For example, in studies addressing subcutaneous adipose tissue metabolism, a superficial epigastric vein can be cannulated [36]. However, the success rate for a skillful person is 50-80% depending on the selection of subjects. Moreover, there are several technical difficulties with the techniques for blood flow measurement like the ^{133}Xe -clearance technique [37]. The interindividual variation is rather high (coefficient of variation up to 20%) and the rate of disappearance is rather slow which makes it difficult to perform fast kinetic experiments.

4.2 MICRODIALYSIS

The microdialysis technique was designed for assessments of the interstitial water in rat brain [38] and was further developed for interstitial fluid sampling in humans and animals [39] in both adipose [40] and muscle tissue [41]. The microdialysis probe is a hollow fiber which functions as an artificial vessel. It can be implanted in the adipose or muscle tissue and it is perfused with neutral solvent. The effluent fluid is collected for analysis of substances of interest. The flow rates usually range between 0.3 and 5 $\mu\text{l}/\text{min}$. At higher flow rates (>0.3 $\mu\text{l}/\text{min}$) the microdialysis only measures a fraction of the concentration of a substance in the interstitial fluid due to incomplete recovery, i.e. the ratio between the concentration of the substance in the effluent fluid and the concentration of the substance in the interstitial fluid. The recovery is dependent on the length of the membrane, on the perfusion speed and on the molecular weight of the sampled substance. In many cases, only the relative change of a substance is of interest, like in comparisons between groups or in a single individual after dynamic perturbation. In order to estimate the true concentration several calibration techniques were developed: zero flow rate protocol, no net flux protocol, and internal reference technique.

The first calibration procedure (zero flow rate method [41]) is based on the assumption that at zero flow rate the interstitial fluid and the perfusate are in complete equilibrium. Thus, the absolute concentration of a substance can be estimated by measuring the concentration at different flow rates (0.5 to 4 $\mu\text{l}/\text{min}$) and applying non-linear regression analysis to extrapolate the interstitial concentration at zero flow rate.

The second calibration procedure (no net flux method [42]) is based on the principle that measurement of substrate in the samples with differing concentrations of that substrate in the perfusate will enable estimation of the absolute concentration in the interstitial fluid at the point where net exchange

across the catheter is zero (the perfusate and the interstitial concentration are equal). The in- and outgoing concentrations are measured and the values can be used in a linear regression analysis.

Both the zero flow rate and the no net flux estimations are indirect and the methods are very time consuming. Hence, these techniques can be used only for the estimation of steady-state values and are not suitable for rapid kinetic experiments.

The addition of labeled substance is used in the third calibration procedure: the internal reference calibration technique [43]. Assuming that the efflux of labeled substance equals the relative flux of substrate, this method enables the estimation of the recovery as the ratio: (labeled substance in the perfusate — labeled substance outflow) ÷ (labeled substance in the perfusate).

Microdialysis is suitable to measure small water-soluble substances like amino acids, glucose, lactate, pyruvate or glycerol. The membrane usually has a molecular cut-off point of about 20 kDa. Large proteins can be determined if a membrane with a higher cut-off point is used (100 kDa). However, the equilibrium time is very long in this type of case and the sample volumes are often very small. Often the sample volume is small and there is incomplete recovery of the compound of interest. Hence, it is necessary to use very sensitive separation and detection techniques like GC-MS, or high-performance liquid chromatography (HPLC) (see also chapter 7). The microdialysis technique was successfully applied to the measurement of glucose [40, 45], lactate, glycerol [41], and amino acids [41] in human adipose and muscle tissue, for long-term continuous monitoring in subjects with insulin dependent diabetes mellitus (IDDM) [44] as well as for the measurement of compounds in rat brain [46]. It is a complementary technique and can be also combined with other regional or systemic measurements.

4.3 MICROPERFUSION

The concept of negative pressure in the free tissue fluid using chronically implanted porous capsules was developed more than 30 years ago [47]. Originally, this capsule method and shortly after its development, the wick catheter technique were used for acute measurement of the subcutaneous tissue fluid pressure. Later, chronically implanted porous teflon cylinders [48] and the wick technique [49] were also applied for interstitial tissue fluid sampling. However, the invasiveness of these techniques prevented their widespread use in humans for obtaining reproducible sampling probes *in vivo* from the extravascular space and determination of an analyte of interest.

Recently, an open flow microperfusion method for continuous adipose tissue fluid sampling and on-line *ex vivo* monitoring of glucose concentration was developed and evaluated in humans [50]. The method combined a double lumen catheter with perforated outer lumen and an extracorporeal sensor cell. By applying a negative pressure gradient between the inner lumen and the outer lumen of the catheter it was possible to continuously sample interstitial fluid from the abdominal adipose tissue. The porous nature of the catheter allowed the interstitial fluid and molecules and ions to pass through and consequently, to analyze the sampled fluid extracorporeally for glucose [50] or lactate [51]. Like microdialysis, under most circumstances this method measures only a fraction of the true interstitial concentration due to the incomplete recovery. Basically, the same calibration methods (no net flux protocol, zero flow rate protocol, internal reference technique) can be applied. Additionally, a fourth calibration procedure was developed, the so-called ionic reference calibration [50]. This procedure is based on the concentration measurement of small ions (Na^+ or K^+) in the sampled fluid as reference to calculate the recovery as the ratio (ion concentration in the sampled fluid) \div (ion concentration in the interstitial fluid). The basic assumption for this calibration procedure is that the interstitial concentrations of Na^+ and K^+ is constant and known (140 and $4 \text{ mmol}\cdot\text{l}^{-1}$, respectively).

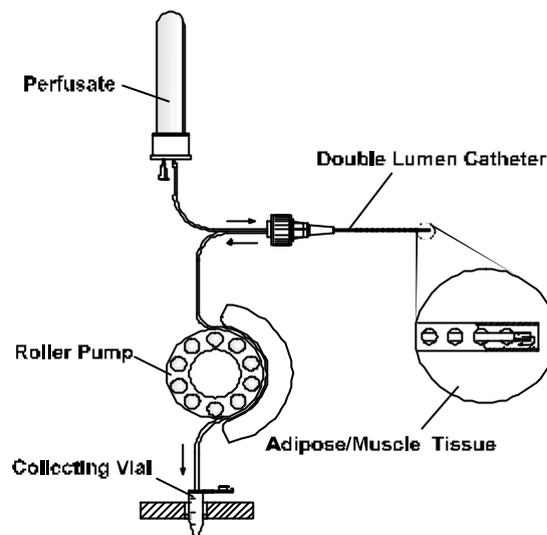


Fig. 6: Schematic representation of the system for continuous *in vivo* interstitial fluid sampling [52]. The double lumen catheter is placed in the abdominal adipose tissue and a negative pressure gradient is applied between the inner and the outer lumen of the catheter using a roller pump (flow rate of $0.5\text{--}5\ \mu\text{L}\cdot\text{min}^{-1}$). The perfusate solution equilibrates with the interstitial tissue fluid and is transported from the outer lumen to the collecting vial.

The major advantage was achieved when the microperfusion technique was modified [52] to enable *in vivo* sampling of molecules with larger molecular weight from the interstitial space in adipose and muscle tissue (Fig. 6). Simplifying the system, the use of larger catheters and precision pumps enabled sampling of volumes sufficiently large for standard laboratory analysis of various molecules. Using this system, the local concentrations of leptin, FFA (bound to albumin and other fatty acid binding proteins) and $\text{TNF-}\alpha$ were assessed in lean and obese women [53]. Although other molecules of interest, like insulin growth factor 1 (IGF-1), IGF binding proteins, insulin, or amylin have not yet been measured, the microperfusion techniques would most likely be capable of sampling these molecules. Also, the application of various agents for local metabolic studies without causing systemic effects is possible.

Furthermore, a smaller catheter which was used for on-line monitoring of glucose, enabled interstitial tissue fluid sampling from hindlimb muscle tissue in conscious rats as shown recently [54]. Using mannitol as an extracellular marker, the intracellular glucose concentration was estimated with this technique [54]. Thus, this technique has great promise for future *in vivo* assessment of metabolism. The major features of both microdialysis and microperfusion methods are summarized in Table 1.

Table 1. Features of the microdialysis and microperfusion technique

MICRODIALYSIS	MICROPERFUSION
Restricted to small and water-soluble molecules	Enables sampling of small molecules and macromolecules
Can be used in brain, muscle, and adipose tissue in humans and animals	Can be used in muscle and adipose tissue in humans and animals
Allows long-term continuous sampling	Allows long-term continuous sampling
Local manipulation of metabolism is feasible	Local manipulation of metabolism is feasible
Minimal tissue damage	Minimal tissue damage
On-line analysis of ions and metabolites (continuous biochemical monitoring)	On-line analysis of ions and metabolites (continuous biochemical monitoring)

4.4 NMR SPECTROSCOPY, PET

NMR and PET are techniques for the non-invasive assessment of regional metabolism. Both techniques have been used to study the metabolism of well-defined regions of human tissues and organs *in vivo* for the last 15 years and there is an increasing number of studies using these techniques.

Based on the NMR phenomenon, NMR spectroscopy provides information about cellular energetics, membrane turnover, neuronal function, neurotransmitter activity and the fate of anaesthetic agents and drugs [55]. Certain atomic nuclei, such as ^1H , ^{31}P , ^{13}C , ^{19}F , and ^{15}N have a spin greater than zero and can be excited by irradiation with radiofrequency energy. During relaxation following excitation, radiofrequency signals can be detected and resolved into a frequency spectrum by the Fourier transformation. The relative frequency position of a metabolite signal (chemical shift) is influenced by the chemical environment of the nucleus. This technique is intrinsically insensitive and only compounds near the millimolar range can be detected *in vivo*. Most of the research on glucose metabolism uses ^{31}P , ^{13}C , and ^1H NMR spectroscopy.

The ^{31}P nucleus has been used to observe phosphocreatinine, adenosine triphosphate and inorganic phosphate. More important, ^{31}P spectra can be used for studying G6P in muscle and liver [56, 57].

^{13}C NMR spectroscopy was developed over the past several years and has added new information on glycogen metabolism in muscle and liver in both animals and humans. The major advantages of ^{13}C spectroscopy compared to muscle biopsy is the noninvasivity and the improvement in time resolution. The concentration of glycogen within an individual muscle or liver can be determined at several time points and consequently, the rates of glycogen synthesis or breakdown can be calculated from the slope of the concentration curve [58]. Glycogen concentrations are obtained by comparing each human spectrum with a spectrum

that is obtained from an external standard solution. Furthermore, ^{13}C NMR can be used to observe glycolytic fluxes by measuring $[1-^{13}\text{C}]$ glucose incorporation into $[3-^{13}\text{C}]$ lactate and $[3-^{13}\text{C}]$ alanine [59]. Recently, ^{13}C NMR spectroscopy was also applied for studying glucose uptake and metabolism in the brain [60]. NMR enables measurement of the cerebral oxygen consumption, brain glucose uptake, and ^{13}C label entering the glutamine and γ -aminobutyric acid (GABA). Since glutamate can be transformed into glutamine only in the glial cells and into GABA in the neurons, glial and neuronal metabolism can be separated which might provide insight into their interaction.

Intra-myocellular lipid droplets are located in the cytoplasm in contact with mitochondria and represent an energy supply during fasting. Intra-myocellular lipid in human muscle can be determined by means of localized ^1H NMR spectroscopy [61]. Hence, NMR spectroscopy has the potential to measure both metabolites which are important for energy store, namely glycogen and lipids. It is noteworthy that, as opposite to ^{13}C and ^{31}P NMR spectroscopy, localized ^1H NMR spectroscopy for lipid measurement does not require strong magnetic fields and can be done on a standard 1.5 T system. Such systems are now routinely used in clinical settings.

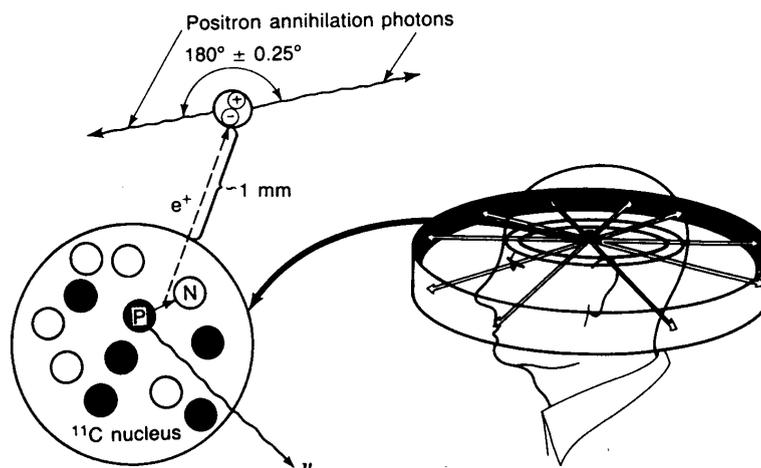


Fig. 7: Principle of PET. Short-lived tracer (positron emitter) is infused in the body of the patient. During the annihilation process of the positron two opposite photons are generated which enable the precise localization of the tracer.

The [^{18}F]fluorodeoxyglucose method is a method for quantitative measurement of regional cerebral glucose utilization which was adapted for use with PET (Fig. 7) [62, 63]. This short-lived tracer is minimally and slowly metabolized after phosphorylation and can be used for the assessment of glucose transport and phosphorylation in human heart [64] and skeletal muscle [65]. After bolus injection of the tracer, serial PET imaging can define uptake curves from which kinetic rate constants for transport and phosphorylation can be determined.

Both, NMR spectroscopy and PET imaging are promising techniques for *in vivo* metabolic studies. However, the major limitation nowadays is the availability of the instruments. NMR spectroscopy devices with whole body bore magnets and fields >2 T or PET scanners are expensive and rare. Both techniques are presently not considered essential for routine diagnosis, but they might prove valuable for prognosis in some circumstances.

5 MATHEMATICAL MODELING

Whole-body glucose metabolism and its hormonal control is a complex system and its quantitative description is a difficult task. Due to inherent nonlinearities, inter- and intraindividual variability, as well as time-varying system behavior, a mathematical model of the whole-body glucose metabolism can only be an approximate description of the real system. Even if an accurate model of the glucoregulatory system were given, obvious practical and ethical limitations would prevent sufficiently precise parameter estimation for an individual subject. Therefore, simplifications and abstractions are necessary in order to be able to quantitatively answer a specific question.

The majority of the presently used models are based on the assumption of compartmentalization, i.e. the substance of interest has homogenous concentration in a given space. For the mathematical modeling of metabolic and endocrine systems, a control engineering-based framework was developed and established [66]. In this framework, the applicability of a model is tested in a two step procedure. After the definition of the structure of the model, the *a priori* or theoretical identifiability of the model is tested, i.e. the question is answered if the model parameters can be theoretically identified. In the second step, the *a posteriori* or practical identifiability of the model is tested, i.e. are the model parameter identifiable from a given experimental data set. Only models which are identifiable can be used for quantitatively answering a specific question. One class of such models is the class of so-called minimal models: the cold minimal model [67], the hot minimal model [68] and the new minimal model [69]. Models which are not identifiable are used for qualitative studies, like simulation studies on closed-loop control of glucose [70-72], or pharmacokinetic studies on insulin absorption [73].

Another important mathematical technique is the reconstruction of endogenous fluxes. Since endogenous fluxes like insulin secretion or hepatic glucose production are either not accessible to measurement or accessible under very invasive conditions, other approaches had to be developed to calculate these fluxes. This is a classical inverse problem and the approaches for the solution are based on the solution of the Fredholm integral of first order, or deconvolution.

In the following section the minimal models for glucose utilization and the methods for reconstructing endogenous fluxes are outlined. Both, the minimal models and the methods for flux reconstruction are now widely used. These mathematical techniques are noninvasive or less invasive than alternative methods, simple in terms of experimental effort, and due to the availability of inexpensive computational power they gain increasing popularity within the scientific community.

5.1 MINIMAL MODELS

Identification of the earliest metabolic changes that forewarn NIDDM onset has long been a goal of physicians and scientist. Due to the absence of genetic markers, tolerance to oral (OGTT) or injected (intravenous glucose tolerance test (IVGTT)) glucose load is an important test for the evaluation of the metabolic function. However, glucose tolerance is determined by complex interaction of insulin secretion, hepatic and peripheral insulin action and other factors. Standardized tests, like the glucose clamp technique, are not suitable for population studies and can be performed only in a limited number of specialized centers. Thus, a relatively simple approach for quantitative estimation of the glucose tolerance would be of great benefit.

The approach based on minimal modeling and IVGTT was developed almost 20 years ago [67] and was later improved and modified also for labeled glucose [68]. The minimal model was based on a highly simplified interpretation of glucose action and the actions of insulin to normalize blood glucose after glucose injection (Fig. 8).

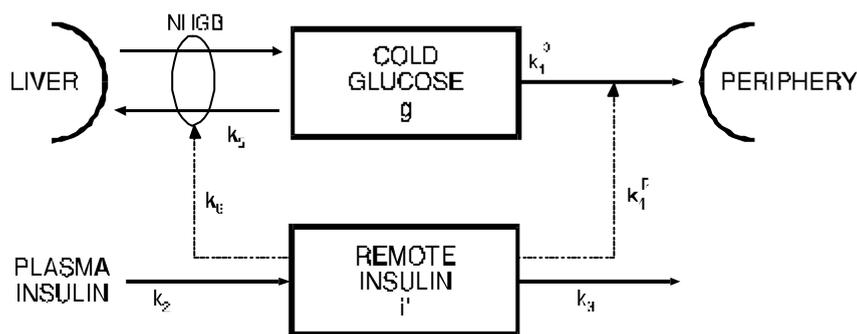


Fig. 8: Minimal model of glucose disappearance. Plasma insulin is thought to enhance glucose utilization in insulin-sensitive tissues by a remote action, presumably by interstitial insulin. NHGB: net hepatic glucose balance, i.e. the difference between glucose production and uptake in the liver. Cold glucose is unlabeled glucose. Note, that it is not necessary to model the plasma insulin kinetics.

The model was made identifiable by lumping certain parameters. The equations for the identifiable model are:

$$\begin{aligned}\frac{dg}{dt} &= -(p_1 + x)g + p_1 g_b \\ \frac{dx}{dt} &= -p_2 x + p_3 (i - i_b)\end{aligned}$$

where g and g_b are the plasma glucose and the basal plasma glucose concentrations ($\text{mg}\cdot\text{dl}^{-1}$), x the remote insulin (min^{-1}), i and i_b the plasma insulin and basal plasma insulin concentrations ($\mu\text{U}\cdot\text{ml}^{-1}$), and p_1 , p_2 , and p_3 constant parameters (min^{-1} , $\text{ml}\cdot\mu\text{U}^{-1}\cdot\text{min}^{-2}$, min^{-1}). Assuming a euglycemic hyperinsulinemic clamp and using the above equations, the insulin sensitivity (S_I ($\text{min}^{-1}\cdot\text{ml}\cdot\mu\text{U}^{-1}$)) and glucose effectiveness (S_G (min^{-1})) can be derived from the estimated parameters as:

$$\begin{aligned}S_I &= \frac{p_3}{p_2} = \frac{k_2(k_4^L + k_6)}{k_3} \\ S_G &= p_1 = k_1^P + k_5\end{aligned}$$

The minimal model has gained increasing popularity and was further modified for labeled glucose injection. This hot minimal model [68] enabled the calculation of endogenous glucose (as opposite to the exogenous) and in theory, also the calculation of hepatic glucose production [68]. However, recent studies have shown that some model assumptions may not be entirely correct [74-77]. It was shown that the glucose effectiveness is overestimated [74] and that the insulin sensitivity is underestimated [75]. These results can be attributed to the approximation of the glucose pool by a single compartment [76-77].

The IVGTT as used for the minimal model analysis, can be also used to test the β -cell function. Measurement of plasma C-peptide concentration and using parametric models of insulin secretion [78] enables estimation of the insulin secretion time-course and parameters of first- and second-phase sensitivity to glucose. However, the knowledge of individual C-peptide kinetics is essential,

which requires a separate experiment [78]. The use of a two compartment model and standard values for C-peptide kinetics enables accurate estimation of the secretion parameter. Thus, using a simple experimental procedure and mathematical modeling techniques, indexes of β -cell function and glucose disposal can be determined.

The development and the diversity of the minimal models over the past 20 years demonstrate the applicability and the inherent limitations present in every model, experimental or mathematical. The structure of the models is based on the state-of-the art knowledge about a given system. Generation of new experimental data, acquiring further knowledge about the metabolic mechanisms force to change the structure or, given a structure which cannot be explained by the available information necessitates additional experimental data. Thus, model development and design of experiments are two processes which go hand in hand.

5.2 RECONSTRUCTION OF ENDOGENOUS FLUXES

Alteration of various rates of production and disposal in different pathophysiological states might help in recognizing the development of a disease. However, metabolic fluxes like HGP, glucose disposal, glycolysis, glycogen synthesis and breakdown, lipolysis, or hormone secretion are difficult to measure *in vivo*, especially in non-steady state conditions. For example, a simple model [79] is used to determine HGP during euglycemic hyperinsulinemic clamp, whereas a more sophisticated approach is required to calculate HGP during IVGTT [69]. In few cases, invasive techniques, like the arteriovenous differences combined with blood flow measurement can be used, but in the majority of studies one can measure only certain parameters and have to calculate backward the endogenous flux of interest. It should be pointed out that, from the mathematical point of view, similar problems also occur in NMR spectroscopy, i.e. derivation of a spectrum for a single compound from a sum of observed spectra. In both cases the problem to be solved is related to the class of the so-called inverse problems [80] and special numerical techniques have to be applied to obtain a solution.

In general, only certain compartments are accessible to measurements, like plasma and only recently, interstitial fluid or cerebrospinal fluid. Only the measurement of the concentration of given substance in that compartment is possible and the flux in that compartment has to be determined:

$$Q(t) = \int_0^t R_a(\tau)h(t-\tau)d\tau$$

where $h(t)$ represents the impulse function, $R_a(t)$ the flux into the compartment and $Q(t)$ the mass of substance. Hence, knowing $h(t)$ (either from *a priori* information or calculated from an independent experiment) and measuring $Q(t)$ one should calculate $R_a(t)$. If the system is linear, or in some cases, linear but time variant, the secretion rate can be calculated by solving the integral equation

(deconvolution) [80-83]. However, this type of problem can be ill-conditioned and special techniques must be applied to avoid erroneous results. These techniques are based on a regularization approach [82]. Briefly speaking, the regularization approach requires additional *a priori* information, like smoothness or certain statistical properties of the flux to be reconstructed, in order to obtain a solution.

Approaches based on deterministic and stochastic methods for reconstructing hormone secretion rates were presented recently [84] and we refer the interested reader to this study. Here, we describe briefly only one routinely used method, i.e. the estimation of the insulin secretion rate.

Insulin secretion rate can be measured by deconvolution of C-peptide [85]. C-peptide is secreted in equimolar amounts with insulin, has negligible hepatic extraction and constant peripheral clearance at different plasma concentrations and at varying plasma glucose concentrations. These characteristics were used to develop an approach for accurate estimation of the insulin secretion rate. One can use deconvolution of plasma C-peptide levels and individually derived C-peptide kinetic parameters obtained by analysis of the decay curve after C-peptide administration [86]. Moreover, it was shown that by adjusting the standard C-peptide kinetic parameters for age and obesity the error for the calculated insulin secretion was $< 16\%$ [85]. Thus, it is possible to derive insulin secretion rates by deconvolution of peripheral C-peptide concentration.

6 ANIMAL MODELS

The complexity of pathophysiological disturbances that are associated with NIDDM makes it difficult to study this disease. The disturbances include decreased insulin secretion, decreased action of insulin in skeletal muscle and adipose tissue, and increased hepatic glucose production. These defects are results of a complex interplay between one or more genetic loci and several environmental factors. Due to this complexity, it is difficult to determine which factors are primary versus secondary and to assess the relative importance of the individual components. The application of animal models allows to selectively perturb a complex *in vivo* system and to create selective physiological defects in order to determine their potential contribution to NIDDM. It should be pointed out that, as any model, animal models are balanced by limitations.

Although other animals have been used for studying diabetes [87], due to their efficiency and costs, the small laboratory rodent is the mostly used animal to study diabetes. Recent developments, like the transgenic and gene-knockout approaches enable assessment of the physiological functions of specific gene products. Animal models can be used to assess specific steps in glucose and FFA metabolism which can not be done in humans for obvious reasons. For example, VLDL-TG production can be estimated by using non-ionic detergent [88]. Glucose transport and phosphorylation can be also measured by using ^{14}C deoxy-D-glucose [89] and 3-O ^3H methyl-D-glucose [90]. Animal models also permit study of environmental factors, like diet, drugs, toxins, and infectious agents. New therapeutic agents to prevent or reverse NIDDM must first be tested in animals. Although other alternative approaches, like cell cultures or mathematical modeling can be used to address certain questions, animal models are still a valuable and necessary tool in research studies.

6.1 RODENT MODELS

One system for classification of rodent models of diabetes includes: 1) obese non-insulin-deficient animal syndromes, 2) non-obese animals, and 3) experimental diabetes [87]. The first group is the group with most extensively studied diabetic syndromes corresponding to the commonest form of NIDDM. The well known strains in this group includes the Zucker or fatty rat (*fa/fa*) which exhibits hyperinsulinemia, insulin resistance, obesity but not hyperglycemia, the obese mouse (*ob/ob*) and the diabetes mouse (*db/db*). The availability of these rodent models for human obesity has allowed rapid recent progress in this area. Almost twenty years ago, in parabiosis experiments in which circulatory systems of normal and genetically diabetic mice were united [91], it was shown that the *ob/ob* mouse is unable to produce a sufficient satiety factor to regulate its food consumption, whereas the *db/db* mouse produces a satiety factor but cannot respond to it. It was recently discovered that the mouse *ob* gene encodes leptin [14] which mRNA is identifiable exclusively in white and brown adipose tissue. The mutation in the *ob/ob* mice results in the production of a truncated inactive protein. The *db* gene encodes the leptin receptor and the *db/db* mouse has a mutation that causes the leptin receptor to be incapable of signaling the binding of leptin in the hypothalamus [16].

The BB rat is a non-obese animal model which offers the opportunity to study in detail the early phase of insulin-deficient diabetes mellitus. The metabolic defects of the BB rat are similar to those seen in humans with IDDM. The BB rats are insulin deficient, have increased plasma glucagon and somatostatin, elevated levels of glucose and free fatty acids, and amino acids.

Diabetes in humans and animals may be provoked by stress, infection, or toxins. The use of chemical agents to produce diabetes permits detailed study of the biochemical, hormonal and morphologic events that occur during and after the induction of diabetes [87]. The two agents that have been most extensively

studied are alloxan and streptozotocin. Both are beta cytotoxins and quite safe since the effective diabetogenic dose is 4-5 times lower than the lethal dose. Another experimental model for diabetes is the diet induced insulin resistance. Feeding rats or mice with diets high in fat content causes whole-body and skeletal muscle insulin resistance, hyperinsulinemia, hyperglycemia, and over a longer period diabetes [92]. Feeding animals with diets high in fructose result in an impairment of insulin action in both skeletal muscle and the liver and also lead to hypertriglyceridemia and hypertension [93]. These models can be also used to provide an information on the ability of a specific drug to influence an environmentally induced, nongenetic, form of insulin resistance. For example, it was shown that treatment with troglitazone, a compound within the class of thiazolidinediones, completely prevent the insulin resistance, hypertension, and hypertriglyceridemia induced by a diet high in fructose [94].

Recently, another experimental diabetes approach, i.e. the use of recombinant adenovirus for metabolic engineering of mammalian cells has gained increased interest [95]. A number of gene transfer vectors and techniques based on the properties of DNA viruses have been developed. Adenovirus mediated gene transfer can be performed to mammalian cells not only in culture, but also *in vivo*.

This technique has the potential for elucidating the effects of perturbation of metabolic pathways in whole animals. Viruses exist by the virtue of their capacity to transfer genetic information into host cells. This essential property of viruses is exploited for the creation of efficient gene transfer vector. For example, in a recent study, recombinant adenovirus containing the leptin cDNA was infused in rats which were made chronically hyperleptinemic due to this adenovirus gene transfer [96]. The elevated peripheral leptin levels depleted TG content in liver, skeletal muscle, and pancreas without increasing FFA oxidation.

6.2 TRANSGENIC ANIMAL MODELS

The physiological role of a specific gene can be evaluated using a variety of techniques that alter gene expression *in vivo* either by expression of transgene construct (transgenic) or elimination of endogenous gene expression (knockout). Using these techniques, selective physiological defects can be created and studied to determine their potential contribution to NIDDM. Moreover, these animal models may also be useful to assess the efficacy or determine the mode of action of potential new therapeutic agents. Also the role of newly identified proteins that are implicated in regulation of glucose or FFA metabolism can be further explored. Transgenic mice are now routinely produced [97-101]. The use of other mammals, including rats, is feasible but remains expensive and time consuming.

The transgene construct can be designed to permit overexpression of: 1) a normal or mutant protein, like hormone, receptor or signaling intermediate, 2) antisense RNA that can hybridize with native mRNA and decrease the expression of the native gene product, 3) genes encoding marker proteins or toxins that will identify or destroy specific cell types. The transgene construct DNA is microinjected into the pronucleus of fertilized oocytes and then implanted into foster mothers. The resulting phenotype will reflect the physiological importance of the transgene product and the level of expression of the transgene [100].

The second approach is to eliminate the expression of a specific gene *in vivo* using targeted gene disruption. Gene targeting has primarily been used to create knockout mice, but the technology now exists for engineering animals in whom specific mutants have replaced endogenous genes or in whom the knockout is tissue specific.

The insulin resistance observed in NIDDM patients and their first-degree relatives implicates a possible role for a defect in the insulin signal transduction. There are several signal transduction pathways and the potential site for defects include any

protein in these pathways. Therefore, insulin signal transduction is the most widely studied area using transgenic animals. Some of these recently engineered transgenic animals are the insulin receptor substrate 1 (IRS1) [102] and insulin receptor substrate 2 (IRS2) [103], and the GLUT4 knockout mice [104]. IRS-1 knockout mice exhibited insulin resistance, hyperinsulinemia and glucose intolerance but did not develop frank diabetes whereas IRS-2 knockout mice are diabetic. Mice with genetic ablation of GLUT4 expression are not overtly diabetic, but exhibit only moderate insulin resistance and mild hyperglycemia during an oral glucose tolerance test. Another recent finding is that the absence of TNF- α results in improvement of insulin sensitivity in diet-induced obesity and genetical model (*ob/ob*) of obesity [105]. These partly surprisingly results demonstrate the complexity of the glucose and FFA metabolism. In this context, transgenic animals provide very powerful tools that can be used to assess the potential contributions of candidate loci, genes or mutations to NIDDM-related phenotypes. Cross-breeding of transgenic and knockout mouse strains will allow further insights into insulin action. Moreover, transgenic animal models suggest a potential role for gene therapy. Overexpression of the insulin receptor or GLUT4 results in improved glucose tolerance. However, the benefits and the long-term effects in these models of gene therapy and the application of these techniques in humans need to be explored.

7 ANALYTICAL TECHNIQUES

In biological samples, proteins, nucleic acids, lipids and carbohydrates are often present in very small quantities and sample sizes. Samples obtained using microdialysis and microperfusion or samples obtained from mice, are often limited and require highly sensitive separation techniques. Furthermore, the complexity of carbohydrate solutes and lipids requires many selective analytical techniques and the use of several chemical and biochemical tools and processes. Hence, isolation of a particular molecule is a difficult task that requires methods both for separating molecules and detecting or assaying a specific molecule.

The most widely used physico-chemical methods in the analysis of carbohydrates include NMR, mass spectrometry (MS), gas-liquid chromatography (GLC), polyacrylamide gel electrophoresis (PAGE), liquid chromatography (LC) and high-performance liquid chromatography [106], and more recently high-performance capillary electrophoresis (HPCE) [107]. Because of the inherent hydrophilic nature of carbohydrates, aqueous-based separation methods, like HPLC, PAGE, or HPCE are very suitable for their analysis. Especially HPCE offers several advantages over HPLC and PAGE like higher separation efficiencies, shorter analysis time and small sample amounts. The increasing application of stable isotopes requires MS instrumentation, most frequently combined with GC or HPLC. The major advantage of the GC-MS methodology is that the enrichment and the concentration (by using internal standards) can be determined from several compounds in a single run, thus minimizing sample amounts. Besides these methods, due to their simplicity and cost effectiveness, enzymatic methods are widely used in laboratory analysis [108].

Recently, advances were made also in the field of biosensors, which now enable on-line monitoring of various metabolites. Devices for *ex-vivo* monitoring of glucose in blood [109] and interstitial tissue fluid [50] as well as lactate in these compartments [110, 51] were recently developed and evaluated. These novel

techniques have the potential to greatly facilitate research and routine investigations. These techniques have several advantages over standard laboratory methods: 1) they enable continuous measurement and hence, improve time resolution; 2) blood loss is minimized; 3) the combination of monitoring devices and computer controlled infusion pumps enable the design of automated systems for clamp studies; 4) monitoring in the adipose interstitial fluid is quasi non-invasive and can be performed repeatedly and conveniently over longer time periods; and 5) the use of thin-film biosensor arrays enables simultaneous measurement of various compounds like glucose, lactate, pH, pO₂, pCO₂ and ions [112].

Besides metabolites and their intermediates, studying glucose and FFA metabolism requires also the analysis of macromolecules like enzymes, hormones, DNA or mRNA. The development of techniques for purifying and characterizing proteins and other polymers has led to astounding advances in cell physiology and molecular cell biology. Techniques for purification and characterizing proteins and peptides include centrifugation, PAGE, two-dimensional gel electrophoresis, various chromatographic methods [112], radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA). One of the most powerful methods for detecting a particular protein in a complex mixture is immunoblotting or Western blotting [112]. This three-step procedure combines the resolving power of gel electrophoresis, the specificity of antibodies, and the sensitivity of enzyme assays and is therefore commonly used to separate proteins and identify a specific protein of interest.

The genome analysis, discovery of new proteins and peptides, the production of transgenic animal models, or the synthesis of purified peptides for therapy would not have been possible without the development of a group of techniques, referred to as recombinant DNA technology [112]. This relatively young and dynamic group of techniques became the dominant approach for studying many basic biological processes [113].

For the analysis of specific nucleic acids two techniques are used: the Southern and Northern blotting. A technique for detecting the presence of a specific DNA sequence in complex mixtures is Southern blotting [114] whereas Northern blotting is used to detect a particular RNA in a mixture of RNAs [115]. Polymerase chain reaction (PCR) [116] and the related reverse transcription PCR (RT-PCR) are used to amplify specific DNA sequences. The PCR is so effective at amplifying that DNA isolated from a single human cell can be analyzed for mutation associated with various genetic diseases.

The modern analytical techniques enable further investigation of whole-body and cellular and molecular events in the regulation of glucose and FFA metabolism. The variety of the available techniques and rapid development of new approaches offer many possibilities to address a certain question or to test a hypothesis. Many research centers have nowadays most of the laboratory equipment necessary to perform the desired analysis and also to develop novel and/or more sophisticated methods.

8 SUMMARY

Given the high prevalence of obesity and NIDDM, elucidating the mechanisms underlying these diseases has become a major goal of many research centers. Within the last twenty years, impressive technical improvements, like NMR spectroscopy and PET imaging, developments in analytical techniques as well as modern molecular genetic approaches offered new tools for highlighting pathophysiological abnormalities. The application of some of these methods has the potential not only in understanding the disease, but also in its use as an early predictive marker of outcome. In this review, we presented an overview of some common techniques for assessment of glucose and FFA metabolism *in vivo*. Clearly, presenting all available *in vivo* techniques is beyond the scope of this paper. The presented methods were selected on the basis of current developments and recent break-throughs in this area, as well as on their applicability and widespread use.

To address a specific question, a combination of various techniques might be necessary. For example, to determine if glucose transport or phosphorylation is impaired in the skeletal muscle of diabetic subjects, a method for the estimation of the intracellular glucose concentration *in vivo* was required. A novel method to assess the intracellular glucose concentration using a noninvasive ^{13}C technique was recently developed and evaluated in rats [54]. *In vivo* tissue NMR spectra were acquired during infusion of $[1-^{13}\text{C}]$ glucose and $[1-^{13}\text{C}]$ mannitol under conditions of hyperglycemic hypo- and hyperinsulinemia. The glucose NMR signal that is observed is dependent on the intra- and extravascular concentrations of glucose and the volumes of these compartments. Using mannitol as an extracellular marker and creatine and phosphocreatine as intracellular marker, the intracellular glucose concentration was calculated as the difference between the total glucose concentration (determined from the ^{13}C NMR spectra) and the extracellular glucose. The extracellular glucose was corrected for an interstitial fluid-to-plasma glucose concentration gradient measured by microperfusion.

Hence, in order to answer this specific question, an animal model was combined with systemic (clamp and tracer infusion) and regional (NMR, interstitial fluid sampling) measurements. The samples were analyzed using GC-MS (labeled substances and using internal standards also unlabeled substances), NMR spectroscopy and enzymatic methods.

Supplementary to the presented *in vivo* techniques, *in vitro* methods are inevitable for the investigation of glucose and FFA metabolism. Activity or concentration of enzymes, secretion of substrate from cells in a defined environment, cell differentiation, or altering gene expression can be only studied using *in vitro* techniques. The advantage of these techniques is that the tissue environment can be controlled, often a necessity when hormone regulation is studied. Improved analytical techniques and recombinant DNA technology applied to *in vitro* techniques greatly enriched current knowledge and affected also *in vivo* investigations. Often a combination of *in vivo* and *in vitro* techniques is required especially in this complex and dynamical area.

Variety of techniques covering many areas of life sciences are now available for *in vivo* investigation of glucose and FFA metabolism. Despite the complexity of these metabolic processes, systematic investigations using these techniques have helped acquiring knowledge and further understanding the physiological mechanisms and pathophysiological disturbances. As a consequence of such investigations the development of new therapeutic approaches in the treatment of NIDDM was initiated and led to novel pharmaceutical agents. For example, the metabolic effects of a class of compounds, thiazolidinediones, was investigated in animal models and humans using many of the techniques presented in this review. Thiazolidinediones proved effective in improving insulin sensitivity and glucose tolerance in NIDDM patients [117] in contrast to sulfonylureas, which only enhance insulin secretion [118]. Despite these recent therapeutic developments, given the multiplicity of factors in the predisposition to insulin resistance and the variety of genetic and metabolic abnormalities in NIDDM, these novel agents

would probably provide helpful only in a subset of the NIDDM population. It will be necessary to further investigate and develop other therapeutic options based on traditional and novel approaches, like signal transduction inhibitors, lipolysis inhibitors or gene therapy.

In summary, assessment of glucose and FFA metabolism is a scientific challenge, but with modern techniques, like isotope tracer methods, NMR spectroscopy, microperfusion, transgenic approaches, or PCR, it is possible to further explore the cellular and molecular events involved in the regulation of these metabolic processes. New techniques are under development and will help to fully understand both normal regulation and the abnormalities of glucose and FFA metabolism which accompany NIDDM and obesity, and develop therapeutic agents for the treatment of these complex disorders.

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