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Methodological approaches to assess body-weight regulation and aetiology of obesity

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Obesity, which is becoming one of the major health hazards in developed and developing societies, results from a long-term positive energy balance. Body-weight regulation and stability depend on an axis with three interrelated components: food intake, energy expenditure and adipogenesis, although there are still many unknown features concerning fuel homeostasis and energy balance. Biochemical processes are interconnected, and a separate consideration of each component is often useful for methodological purposes and to achieve a better understanding of the whole system. Thus, many different experimental approaches can be applied by using laboratory animals, cell culture or human subjects to unravel the molecular mechanisms which participate in body-weight regulation. Thus, both *in vitro* (cellular and subcellular models) and *in vivo* methods have dramatically increased our knowledge of weight control. Several strategies in obesity research are reported here, exploiting the opportunities of the molecular era as well as novel whole-body approaches, which will impact on the development of new targets for obesity management and prevention.

Obesity genes: Adipose tissue: Energy expenditure: Body composition

Lessons from molecular and cellular biology and genetics

Application of the concepts and techniques of molecular biology has led to major advances over recent years in the understanding of the mechanisms underlying the regulation of energy balance and obesity development (Trayhurn, 1998). Particular progress has come from the identification, by approaches such as positional cloning, of the mutant genes involved in the initiation of obesity in rodent

models in which the disorder is genetically determined. The localization of mutant genes in these animals has led to the identification of ‘novel’ proteins and previously unrecognized physiological regulatory systems. This progress is especially evident in the case of the 16 000 relative molecular mass hormone, leptin, a mutation in the leptin gene causing the obesity of the *ob/ob* mouse (Zhang *et al.* 1994). The mouse *db* gene and the rat *fa* gene encode

Abbreviation: UCP, uncoupling protein.

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the receptor for leptin, and a mutation in these genes is responsible for the obesity of the *db/db* mouse and *fa/fa* rat respectively (Trayhurn *et al.* 1999). Other single-gene mutation rodent models of obesity have allowed other genes and gene products potentially involved in human obesity, such as carboxipeptidase E and the agouti signalling protein to be identified (Perusse *et al.* 1999).

Homologues of the mouse 'obesity genes' have been reported in human subjects, and searches have been undertaken for mutations in these genes in obese subjects, and for specific polymorphisms, which may be linked to increased body fat or the tendency to fatness (James & Ralph, 1999). Three reports have now identified mutations in the coding region of the leptin and leptin receptor genes in association with severe obesity (Montague *et al.* 1997; Clement *et al.* 1998; Strobel *et al.* 1998). The significance of these findings is not only the presence of mutations *per se*, but the demonstration that the leptin system is physiologically important in the regulation of body fat and energy balance in man as well as in rodents.

Mutations and polymorphisms can be identified in genes which are, or might be, linked with obesity by employing Southern blotting. Expression of a given gene may be assessed in tissue samples *in vitro* by Northern blotting and by other techniques (RNase protection, restriction enzyme methodology, reverse transcription-polymerase chain reaction) for determining specific mRNA (Hesketh & Partidge, 1996; Clarke & Sooja, 1998). These methodologies are able to indicate those tissue(s) expressing a particular gene, which is important in gaining understanding of the physiological function of the encoded protein, but it also enables changes in gene expression in obesity to be determined and whether any alterations are primary or secondary. Furthermore, nutritional, hormonal and neural factors involved in the regulation of specific genes can be investigated as potential mediators of some obesity genotypes. Other methods for detection of mutations are denaturing gradient gel electrophoresis, carbodiimide modification, single-strand conformational polymorphisms, heteroduplex analysis, RNAase or chemical cleavage methods, allele-specific oligonucleotide, ligation detection, artificial introduction of restriction sites and single base primer extension (Beaudet, 1998). Those studies have been complemented by quantitative trait loci trials from cross-breeding experiments as well as by association and linkage studies between candidate genes with the human obesity phenotype through different genetically-based methods and epidemiological studies. The number of genes putatively involved in obesity is increasing very rapidly and now approaches 200, including uncoupling proteins (UCP), β_2 - and β_3 -adrenergic receptors, peroxisome proliferator-activated receptor, adipocyte fatty acid-binding protein, pro-opiomelanocortin, melanocortin-4 and -5 receptors etc. which may be implicated in food intake, adipogenesis or energy expenditure control (Perusse *et al.* 1999; Willett, 1999).

A substantial part of basic knowledge about fat cell function is derived from *in vitro* studies on adipose tissue (Lafontan & Arner, 1996). Isolated fat cells have been extensively used for their rapid metabolic responses. It is an ideal system for the study of metabolite transport, delineation of mechanisms of hormone action and pharma-

cological effects. However, some results may not be representative of the whole adipose tissue mass. Preservation of fat cell integrity depends on the success of the isolation procedure. Recovery of the whole fat cell population of a given adipose tissue requires attention, since large fat cells are fragile and small fat cells may be lost during isolation. Moreover, artifactual leakage of adenosine from isolated fat cells alters metabolic variables (lipolysis, glucose transport). Maintenance of isolated human adipocytes for several days is difficult because of the fragility of the cells, the alteration of hormonal signal transduction and the marked decrease in gene expression. Adipose tissue explants that partly maintain the *in vivo* structure of the tissue and permit long-term culture, although less suitable than isolated fat cells for transport and pharmacological studies, seem to be more satisfactory for the *in vitro* study of some adipocyte functions and the regulation of gene expression.

The dissipation of energy as heat by adaptive mitochondrial thermogenesis may play a role in body-weight control. The UCP family of mitochondrial inner-membrane transporter proteins participate in uncoupling respiration from ATP synthesis (Nicholls & Locke, 1984). The recent identification of novel UCP (mainly UCP2 which is widely expressed and UCP3 which is mainly present in muscle) has attracted new interest, although their role, particularly in human subjects is not clear (Schrauwen *et al.* 1999). Lipid-soluble vitamins, fatty acids, and particularly the retinoids, appear to be involved in the regulation of UCP1 gene expression, which has been the focus of extensive effort (Puigserver *et al.* 1996; Bonet *et al.* 1997).

Lessons from animal studies

Small animals (mainly rats or mice) are very useful for carrying out both nutritional and obesity-related studies. The utilization of metabolism cages for the individual recording of food and water consumption as well as outputs of urine and faeces allows the determination of nutrient balance and the assessment of dietary influences on growth and body composition (Esteve *et al.* 1992). Experimental trials can be performed by using dietary interventions with different energy content and macronutrient composition, including the utilization of highly-palatable foods and cafeteria diets to induce overweight, which provide a model for understanding body-weight regulation. In addition, the use of rodents in metabolism studies greatly expands the methodological approaches possible, since experimental animals can be cannulated under anaesthesia and used for the determination of arterio-venous differences in key organs and for tissue blood flow measurement. Circadian rhythms of individual tissue temperature can be determined by implantation of temperature probes in different tissues. In addition, the research on animal models (mouse or rat) with both lean or obese phenotypes, either due to known mutations (*db/db* and *ob/ob* mice and *fa/fa* rats) or as a result of direct genetic manipulation (a knockout or transgenic animal) of key genes related to body-weight control, can facilitate the study of metabolic or nutritional variables by the direct comparison between the lean and obese individuals (Closa *et al.* 1992; Docherty, 1996).

In addition, energy expenditure determinations by respiratory gas exchange (CO₂ and O₂) in rodents and other animals are valuable tools to investigate short- and long-term changes in organ weights (including fat tissue). Body composition may be measured by different chemical, electromagnetic, ultrasound and other procedures. Furthermore, substrate utilization and fate, as well as metabolic pathways, can be appropriately assessed by using different stable or radiolabelled tracers in order to measure energy metabolism, kinetics and nutrient turnover. Finally, the screening of the role of different neuropeptides or molecules such as cocaine- and amphetamine-regulated transcript, serotonin, leptin, orexins and neuropeptide Y involved in appetite and energy metabolism regulation can be conveniently estimated by using conventional or stereotactic administration, with suitable methods and pharmacological equipment (Schwartz *et al.* 1999). Research on neuroendocrine and sympathetic signals as well as on the hypothalamic control of energy balance has benefitted from the use of suitable animal models (Bray, 1999; Webber & Macdonald, 1999; Williams *et al.* 1999).

Searching for genes differentially expressed in obesity models

RNA fingerprinting, or differential display, is one of the several molecular biology strategies available to detect genes differentially expressed between individuals who have developed diet-induced obesity *v.* control lean individuals. The availability of a rat model for diet-induced obesity allows the comparison of white fat, skeletal muscle and liver tissue samples in order to identify genes that might have an important role in the development of obesity. This approach is considerably more straightforward than other approaches described previously, such as differential screening or subtractive hybridization, and has important advantages; much less sample is needed, it allows the comparison of multiple samples and the simultaneous identification of genes that are either up or down regulated. The most important pitfall is the relatively high number of false positives that can be obtained, which makes careful screening absolutely necessary before further efforts are focused toward any particular sequence identified. To simplify the task of handling the numerous possible positives several alternatives have been proposed, although Northern blot confirmation of differential gene expression is required to obtain a definitive answer (Liang & Pardee, 1998).

RNA fingerprinting, or differential display, has already been applied successfully in a considerable number of studies, resulting in the identification of new genes or demonstrating the role of previously characterized ones in the process under investigation. These studies include adipocyte differentiation (Hu *et al.* 1996), the hypothalamus and skeletal muscle of genetically-obese (*ob/ob*) mice (Vicent *et al.* 1998), and even rodent models (OM and S5B/PI strains) of diet-induced obesity (Lin *et al.* 1998).

An alternative and promising new technology is the oligonucleotide microarray (DNA chips)-based hybridization analysis, which potentially allows rapid and cost-effective screens for all possible mutations and sequence variations in genomic DNA (Hacia, 1999). Technological

advances within the past decade have made possible the application of this technology to medical genetics.

Gene transfer strategies in obesity models

Gene delivery technology presents the major obstacle in the development of vectors which appropriately transfer and express relevant gene products in specific tissues. Thus, the ideal gene-transfer vector would be injectable, targetable to specific sites *in vivo*, non-immunogenic, able to be regulated and maintain long-term gene expression.

In an attempt to investigate some processes involved in obesity, delivery of the leptin cDNA by viral or non-viral vectors in lean and genetically-obese (*ob/ob*) animals has been performed. A single leptin gene injection into the tibialis anterior muscle of mice resulted in a 2-fold increase in serum leptin levels, together with a modest and reversible reduction in food intake and weight gain in *ob/ob* mice (Marti *et al.* 1998). Furthermore, treatment of these animals with a recombinant adenovirus expressing the mouse leptin cDNA (AdRSV-leptin) resulted in a rapid reduction in food consumption and drastic weight loss, which led to a complete, although transient, correction of obesity and diabetes as serum leptin levels decreased after 2–3 weeks (Muzzin *et al.* 1996). Comparing the efficacy of daily injection of recombinant leptin protein with adenovirus-mediated delivery of leptin for obesity treatment, Morsy *et al.* (1998b) found that there is a continuous chronic secretion of leptin mediated by gene delivery *v.* the intermittent bolus delivery and rapid clearance of the daily leptin protein injection. Furthermore, *in vivo* studies suggest that leptin effects are better achieved at the lower steady-state levels achieved by gene therapy. However, adenovirus-mediated *in vivo* gene transfer and expression are limited in part by cellular immune responses to viral-encoded proteins and/or transgene immunogenicity. To diminish these responses helper-dependent vectors have been developed in which the viral protein coding sequences are completely eliminated. These helper-dependent vectors have up to 37 kb insert capacity, are easily propagated in a CAMP response element recombinase-based system, and can be produced to high concentration and purity. In contrast to the liver toxicity, inflammation and cellular infiltration observed with first-generation E1-deleted adenovirus vectors, helper-dependent leptin delivery was connected with a significant improvement in associated safety and toxicity (Morsy *et al.* 1998a). The greater safety, insertion capacity and efficient gene delivery of the helper-dependent vectors represent favourable features for clinical gene-therapy applications.

Moreover, recombinant adeno-associated virus carrying the leptin cDNA were also constructed and the effects characterized after one intramuscular administration in *ob/ob* mice. Contrary to results obtained after adenovirus-based leptin delivery, the weight-reducing effects lasted for at least 6 months and the leptin expression for at least 3–5 months (Morsy *et al.* 1998a). Expression of a marker gene from recombinant adeno-associated virus vectors injected into muscle gradually increased over a period of 4–6 weeks before stabilizing, while adenovirus gene delivery resulted in rapid onset of protein expression which is extinguished within 2 weeks, presumably by immune responses to

adenoviral proteins. The recombinant adeno-associated virus–leptin-treated animals showed normalization of metabolic abnormalities, including hyperglycaemia, insulin resistance, impaired glucose tolerance and lethargy which leads to a long-term correction of obesity and diabetes in the obese (*ob/ob*) mouse model. It is thus encouraging that gene-transfer technology in obesity models (monogenic) is progressing, although substantial research efforts are needed to be able to screen and treat (polygenic) obesity.

Lessons from human studies

Methodologies for the study of human body-weight regulation must be focused on the hypothesis to be tested, and must have the sensitivity and specificity to yield a definitive result.

Assessing energy expenditure and lipogenesis by using stable isotopes

The accuracy and non-invasive nature of stable-isotope-based methods make them ideal for the study of human energy metabolism (Diaz & Marques-Lopes, 1999). The doubly-labelled water method was developed 50 years ago, but nearly 40 years passed before it became a major tool for human nutrition research. This method has been applied extensively to the study of the growing problem of obesity in order to determine the role of energy expenditure and physical activity in weight control. Furthermore, it has provided a new means of validating methods for assessing dietary intake (Schoeller, 1999).

Energy metabolism is regulated by several neuro-endocrine and nutritional factors affecting the macronutrient balance (Tappy *et al.* 1998). Thus, body lipid, carbohydrate and protein accumulation and metabolic fate can be assessed using *in vivo* and *in vitro* methods by measuring the rate of the different metabolic pathways (dynamic aspects), and also the net balance which may lead to nutrient deposition or loss (static aspects). The quantification of nutrient synthesis and breakdown can be performed by using different radioactive or stable isotopes as tracers within different precursors. As an example, fatty acid synthesis can be independently measured by the intravenous infusion of [¹³C]acetate and the application of the mass isotopomer distribution analysis technique (Hellerstein *et al.* 1996). This method uses probability analysis to measure the synthesis of biological polymers. It is based on the mathematical principle that the labelling pattern of a polymer synthesized from a stable-isotope-labelled precursor will conform to a predicted binomial or multinomial expansion. The actual isotope enrichment is measured by GC–mass spectrometry. This method requires that newly-synthesized (labelled) and preformed (unlabelled) components mix in the liver and communicate with plasma VLDL over the period of the isotope infusion. It also assumes that the major *de novo* fatty acid is only a single fatty acid, with minor elongation and/or desaturation processes. Finally, the infused isotopically-labelled acetate should have no physiologically important effect. This methodology can be applied to assess lipogenesis in very different nutritional and physiopathological conditions such

as diabetes, obesity etc. (Martinez & Marti, 1998; Hellerstein, 1999). Of course, other tracers are also valuable means of quantifying nutrient turnover (oxidation, deposition etc.) by using appropriate markers in order to measure the oxidative hierarchy of macronutrients, the whole-body macronutrient balance, glucose–fatty acid interactions and the steady-state of weight maintenance at which the fuel mixture matches the amount and composition of dietary nutrient intake (Jecquier & Tappy, 1999; Schutz, 1999).

Assessing human body composition

In recent years there has been renewed interest in the individual components of body weight. This interest has included developments in the methodology for the study of nutrient partitioning, field methods suitable for large epidemiological studies and independent techniques for the measurements of fat distribution, muscle mass or other elements of body composition. These methods have been extensively reviewed (for example, see Jebb, 1998).

Detailed studies of body composition are essential in a number of areas:

- (1) to define reference standards for body composition during growth and development;
- (2) to define the relationship between body composition, including fat distribution, and long-term health outcomes;
- (3) to measure differences in body composition between individuals or in response to different manipulations in order to further our understanding of the mechanisms regulating nutrient partitioning;
- (4) to test the acute and chronic impact of interventions on body fat mass in both experimental, laboratory-based and community studies.

For epidemiological studies the emphasis is on methods which are capable of ranking individuals accurately and those which have minimal observer error. Here, new developments which make current methods simpler, cheaper or more practical will become increasingly important. For more detailed metabolism studies the problem lies not so much in the methods themselves but in the models used to describe the composition of the body. Classical two-compartment techniques, which divide the body into fat and fat-free tissue are gradually being replaced by four-compartment models comprising water, bone mineral, fat and protein, or even further sub-compartments (Fuller *et al.* 1992).

Unfortunately, at the present time even the best methods or models are not always of sufficient sensitivity or specificity to yield definitive conclusions in some research studies. Even the ‘reference’ methods such as densitometry, total body water or dual-energy X-ray absorptiometry are unable to measure body fat to much better than ± 1 kg, and ‘field’ methods such as skinfold thicknesses or bioelectrical impedance may be considerably worse (Jebb *et al.* 2000). However, changes in body composition can be estimated more accurately using classical measurements of energy balance. Using continuous measurements of substrate balance within the confines of a whole-body calorimeter, energy intake and expenditure can be measured precisely, to

within ± 9 g fat/d and 20 g carbohydrate/d (Jebb *et al.* 1993). Accordingly, it is possible to examine the precise impact of a variety of interventions over very short periods of time and in much smaller groups of subjects than is necessary when using other methods (Poppitt *et al.* 1997).

In spite of their limitations, the careful application of current methodologies to measure body composition *in vivo* and the development of other methods, including an imaging technique (Sjostrom, 1991), targeted at specific research questions will be a critical element in the development of our understanding of body-weight regulation.

Strategies to investigate human adipose tissue functions

Adipose tissue is a heterogeneous metabolic organ (Havel, 1999). A number of *in vivo* and *in vitro* techniques are available, with different advantages and limitations, for investigating various aspects of adipose tissue metabolism. Tracer methods, using glycerol or fatty acids labelled with stable or radioactive isotopes, allow the evaluation of whole-body metabolic rates, but it is necessary to utilize complex multicompartmental models to derive equations reflecting physiological events, although they do not give information about regional adipose tissue metabolism. Measurements of arterio-venous concentration differences across subcutaneous adipose tissue, combined with measurements of adipose tissue blood flow, allow measurement of uptake and output of metabolites or any other exogenous substances.

In human subjects a new technique has been developed recently to quantify substrate turnover in the abdominal subcutaneous adipose tissue *in vivo* after cannulation of the left superficial epigastric vein for sampling adipose tissue venous blood (Frays *et al.* 1993). The method, which is relatively difficult and time-consuming, is restricted to human subcutaneous fat deposits. The microdialysis technique is a promising *in vivo* method which allows continuous sampling and manipulation of the interstitial space of adipose tissue without influencing whole-body function (Lafontan & Arner, 1996). Microdialysis measures the concentration of a particular substance in the interstitial space. The technique gives information on subcutaneous fat deposits, allowing long-term continuous measurements and local manipulation of adipose tissue metabolism, which is essentially suitable for measuring small and water-soluble molecules which easily cross the dialysis membrane (the usual cut-off point of membranes is 20 000 Da). It is possible to estimate the true concentration of a substance in the extracellular space of adipose tissue with microdialysis when using a calibration technique to determine the recovery rate from the dialysis probe (depending on the length of the dialysis probe and the perfusion speed). It is also possible to indirectly estimate local blood flow in adipose tissue by microdialysis (Galitzky *et al.* 1993).

In vivo regulation of muscle and adipose tissue gene expression

In spite of body-weight regulation and adaptation to environmental factors such as nutrition and lifestyle by

changes in gene expression, obesity and other complications may occur (Lean, 1999). In pathological states, such as obesity or non-insulin-dependent diabetes mellitus, defects in this regulation could occur and could explain inter-subject variations in the response to different diets and obesity occurrence. However, the study of such regulation in human subjects has been limited by the lack of access to different tissues. The development of micro-methods such as the reverse transcription-competitive polymerase chain reaction allows the measurement of mRNA concentrations in very small amounts of tissue that could be safely obtained (Auboeuf *et al.* 1997). Thus, specific assays have been designed in order to quantify mRNA from numerous target genes involved in insulin signalling (insulin receptor, insulin receptor substrate 1, phosphatidylinositol-3-kinase), in glucose metabolism (glucose transporter 4, glycogen synthase, hexokinase II) and in lipid metabolism (hormone-sensitive lipase, lipoprotein lipase, leptin and other regulatory processes (tumour necrosis factor, plasminogen activator inhibitor-1 etc.).

In this context, the effects of insulin infusion, energy restriction and lipid infusion were studied in control and obese subjects, and in subjects with non-insulin-dependent diabetes who were submitted to muscle (*vastus lateralis*; 60–80 mg) and periumbilical subcutaneous adipose tissue biopsies before and after the nutritional intervention in order to quantify changes in gene expression. Defects in phosphatidylinositol-3-kinase, hormone-sensitive lipase and UCP2 gene expression regulation were found, which may help us to understand the physiopathology of non-insulin-dependent diabetes (Laville *et al.* 1996; Andreelli *et al.* 1999). Other genes potentially involved in human thermogenesis (UCP, β_3 -adrenergic receptor, peroxisome proliferator-activated receptor- γ coactivator-1) and adipocyte differentiation (peroxisome proliferator-activated receptor adipocyte fatty acid-binding protein) have been recently identified and are under study.

Further assays are now under development, combining studies in human subjects with *in vivo* and *in vitro* systems (Yanovski & Yanovski, 1999) in order to assess the mechanisms involved in body-weight regulation and obesity (Martinez, 1999). This assay development is of a major importance in terms of clinical nutrition, and for public health, since the prevalence of obesity is increasing at alarming rates in many populations (Pi-Xunyer, 1999).

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