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Using Gene Knockout and Transgenic Techniques to Study the Physiology and Pharmacology of β_3-Adrenergic Receptors

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MANY factors contribute to the regulation of energy balance: energy expenditure, food intake, brain, fat cells, hormones, etc. Because these various components are all interconnected, it has been difficult to determine the relative contribution of each to the pathogenesis of obesity. Mouse genetics offer two major tools useful for identifying the molecular mechanisms which regulate body fat content (Fig. 1). One general approach, positional cloning, involves going from an obese mouse to the responsible genetic defect. Over the past few years this approach has yielded much valuable information. The second general approach, gene targeting and transgenics, involves creating genetic mutations in mice, and then determining the effects of these mutations on regulation of total body fat content. This approach has also yielded significant advances.

During the past few years, all of the mouse single gene mutations which produce obesity have been identified (Table 1, reviewed in ref. [1]). The *ob* mutation causes deficiency of leptin, an adipocyte secreted hormone which communicates the status of fat stores to the brain, the *db* mutation disrupts the receptor responsible for transducing the leptin signal in the brain, the *fat* mutation abolishes carboxypeptidase E activity, the *tub* mutation disrupts a hypothalamic protein of unknown function and the A^y mutation leads to overexpression of agouti, an inhibitor of melanocortin receptors.

The mouse genome can be manipulated in two ways [2]. Transgenics involves the injection of DNA constructs into mouse zygotes to create gain-of-function mutations. Gene targeting, on the other hand, typically involves the creation of loss-of-function mutations in which genes are deleted, or "knockout out" from the genome. We have used both of these approaches to explore the regulation of energy expenditure in mice. One experiment involved the transgenic ablation of brown adipose tissue to create mice with diminished brown fat [3]. The other experiment involved knockout of the β_3-adrenergic receptor gene to create mice which completely lack β_3-ARs [4].

Overview of Energy Balance, White Adipocytes and Brown Adipocytes

Shown in Fig. 2 is a simplified view regarding the regulation of body fat content, and the role of leptin [5]. Naturally, this is an oversimplification. Triglyceride is stored in white fat. Leptin is secreted from white fat in a manor that is proportional to the size of fat stores. Secreted leptin then communicates the status of fat stores to the brain. The brain then adjusts food intake and energy expenditure in order to maintain normal body fat stores. The brain controls energy expenditure, at least in part, via the sympathetic nervous system

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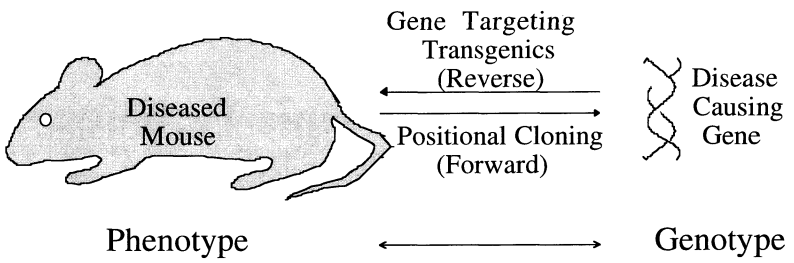

Fig. 1. Using mouse genetics to study obesity. For explanation, please see text.

Table 1. Cloning of mouse obesity genes

Mutation	Defect
ob	Leptin deficiency
db	Leptin receptor defect
fat	Carboxypeptidase E mutation (hormone processing defect)
tub	Hypothalamic protein of unknown function
A^y	Overexpression of agouti (Inhibition of melanocortin-4 receptor)

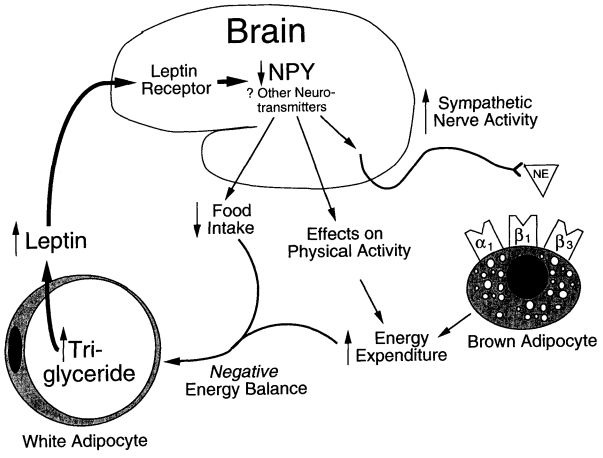

Fig. 2. Feedback loop controlling body fat stores. For explanation, please see text.

which innervates brown adipose tissue. Sympathetic stimulation of brown at makedly increase energy expenditure [6].

White and brown fat are similar in that they both contain triglyceride and express many genes which are involved in the handling of triglyceride. However, white and brown fat are completely different in function [6]. White fat stores calories while brown fat expends calories. Histologically, brown fat cells are smaller, and they tend to store lipid in a multilocular fashion. Also, brown adipose tissue is highly vascular while white adipose tissue is not. Brown adipocytes are also unique in that they have abundant mitochondria, a feature which is important for the expenditure of calories. Perhaps the most distinguishing feature of brown adipocytes, is that they uniquely express the mitochondrial protein called uncoupling protein or UCP [7, 8] (see Fig. 3).

Fuels are oxidized by means of the electron transport chain, creating a proton electrochemical potential gradient. Normally, protons descend this gradient via ATP synthase, coupling synthesis of ATP to fuel oxidation. However, if no work has been done, little ADP exists and protons cannot re-enter via ATP synthase. The proton gradient increases, electron transport slows and fuel oxidation decreases. Thus, in the absence of performing work, cells do not oxidize fuels. Fuel oxidation is "coupled" to the performance of work. Uncoupling protein, or UCP-1, causes the proton gradient to collapse by exporting free fatty acid anions which then function as cycling proton carriers [9, 10]. In essence, UCP allows FFAs to function like dinitrophenol, a classic mitochondrial uncoupler. In the uncoupled state, brown adipocytes oxidize fuels unrelated to the performance of work, the by-product being the generation of heat. Thus, stimulated brown adipocytes can expend enormous amounts of calories.

Recently, a new molecule has been identified, UCP-2 (Ricquier *et al.* GenBank Accession number U69135). It is a homologue of UCP-1 that is likely to function like UCP-1, given its amino acids similarity to UCP-1 (56% identity). However, unlike

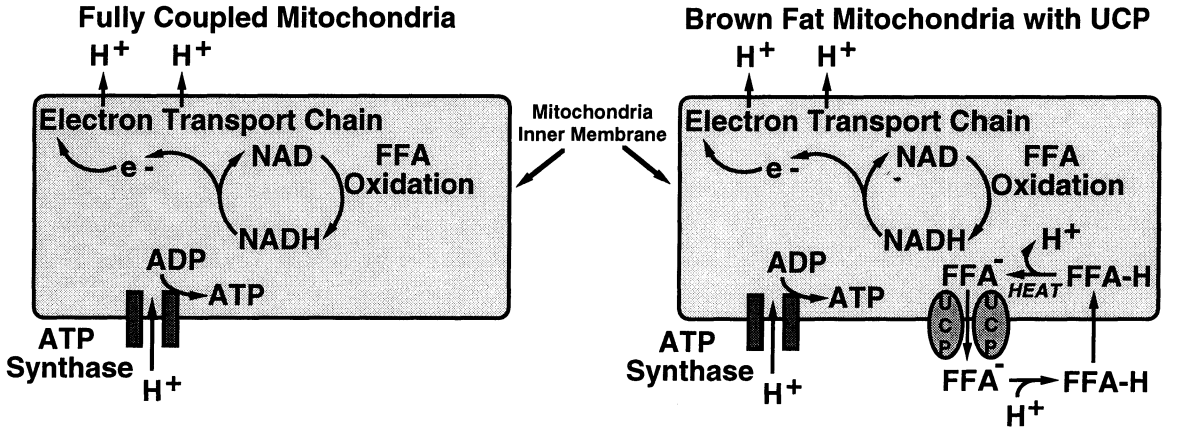

Fig. 3. Fully coupled mitochondria (left panel) and brown fat mitochondria uncoupled by UCP (right panel). For explanation, please see text.

UCP-1 it is widely expressed in mice and humans. Therefore, UCP-2 is likely to be an important determinant of total body energy expenditure. The role of UCP-2 in physiology and disease is presently under intense investigation.

Transgenic Ablation of Brown Adipose Tissue

Some time ago we set out to assess the physiologic significance of brown fat by generating transgenic mice which have decreased brown fat [3]. To do this, the UCP-1 promoter, which is only expressed in brown fat, was used to drive expression of diphtheria toxin A-chain. Diphtheria toxin A-chain then kills brown adipocytes, thus creating mice with decreased brown fat. Of significance, mice with decreased brown fat develop obesity, strongly supporting the view that brown fat plays an important role in resisting the development of obesity in mice. More recently, we have demonstrated that transgenic mice with decreased brown fat have increased susceptibility to diet-induced obesity [11], thus supporting the hypothesis that brown fat protects against obesity caused by calorically dense diets.

Knockout of the β_3-AR Gene

β_3-ARs are found predominately on brown and white adipocytes in rodents. Brown adipocyte activity is controlled by the brain via the sympathetic nervous system. It is possible that mutations in the human β_3-AR could contribute to the development of obesity and there have been recent publications on this topic [12–14]. Finally, pharmaceutical companies have generated agonists which selectively stimulate β_3-ARs, and these β_3-agonists are potent anti-obesity drugs in rodents [15–17].

In order to address a number of issues, we have made gene knockout mice lacking β_3-ARs. To do this we have used the homologous recombination approach [4]. A targeting construct was generated in which the β_3-AR gene was rendered nonfunctional. This construct was then used to create β_3-AR gene knockout mice which lack intact β_3-AR mRNA and functional β_3-ARs. In general, these mice are phenotypically normal except for a small increase in total body fat content. If it is true that β_3-ARs play an important role in controlling brown fat activity, then one might have expected β_3-AR knockout mice to be markedly obese, which was not the case. Alternatively, other gene products might have compensated for the absence of β_3-ARs. Since β_1 and β_2-ARs respond to the same endogenous ligands and utilize the same signaling pathway as β_3-ARs, it is conceivable that β_1- and/or β_2-ARs might have compensated for the absence of β_3-ARs. Indeed, we noted that β_1-AR mRNA was significantly upregulated in brown adipose tissue of β_3-AR gene knockout mice. Upregulation of a related family member, the β_1-AR, in response to knockout of the β_3-AR, strongly implies that β_3-ARs play an important role in controlling brown

adipose tissue function.

Acute treatment of mice with a β_3-selective agonist produces the following effects: 1) a 2-fold increase in energy expenditure, 2) a 50–100 fold increase in serum insulin levels, and 3) a 45% reduction in food intake. While the effects on energy expenditure are almost certainly mediated by β_3-ARs, the effects on insulin levels and food intake are less certain. To determine the role of β_3-ARs in mediating each of these response, control (+/+) and knockout (−/−) mice were treated with CL, a β_3-selective agonist. If the response to CL disappears in knockout mice, then it is assumed that that response is mediated by β_3-ARs.

Effect of CL on oxygen consumption

CL increases the oxygen consumption of normal mice by about 2-fold. This is a rather dramatic increase in energy expenditure and demonstrates the marked anti-obesity action of these agents in rodents. No effect was observed in knockout mice. Thus stimulation of oxygen consumption by CL is mediated entirely by β_3-ARs.

Effect of CL insulin levels

In normal mice, insulin levels dramatically increase by 50–100 fold following the acute administration of CL [4, 18], or other β_3-AR selective agonists [19]. In knockout mice, no effect was observed. Thus, stimulation of insulin secretion by CL is mediated entirely by β_3-ARs. However, the mechanisms for this response is not known. Of note, β_3-ARs are not thought to be present in pancreatic islets. Thus, it is possible that β_3-ARs on adipocytes somehow mediates this response.

Effect of CL on food intake

In normal mice, acute CL treatment suppresses food intake by about 45%. No effect was observed in knockout mice. Thus, inhibition of food intake by CL is mediated exclusively by β_3-ARs. However, the mechanism for this effect is also unknown.

To summarize, acute treatment with β_3-selective agonists produces the following responses: 1) a 2-fold increase in oxygen consumption, 2) a 50–100 fold increase in insulin levels, and 3) a 45% inhibition of food intake. We have shown that each of these responses are mediated exclusively by β_3-ARs. Questions to be addressed include: 1) What is the role of *adipocyte* versus *non-adipocyte* β_3-ARs in mediating each of these responses?, and 2) What is the role of *white* versus *brown* adipocyte β_3-ARs in mediating each of these responses? To answer these questions, we have created mice which express β_3-ARs selectively in white and brown adipose tissue, or in brown adipose tissue only. These animals will be used in the future to delineate the precise the role of β_3-ARs in these sites in mediating each of these responses.

In a related series of experiments, we have also created mice that express human but not murine β_3-ARs. Prior studies have documented that rodent and human β_3-ARs respond differently to a number of β_3-selective agonists. Therefore, convenient animal models suitable for *in vivo* testing of β_3-selective agonists do not exist. To address this issue, we have transgenically introduced 74 kb of the human β_3-AR genomic locus into β_3-AR knockout mice. These animals express human but not murine β_3-ARs selectively in brown adipose tissue. "Humanized" β_3-AR mice should assist in the evaluation of effective β_3-selective agonists. In addition, these animals with provide a basis for investigating promoter/enhancer elements which confer tissue specificity on human β_3-AR gene expression.

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