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Strategies for assessment of botanical action on metabolic syndrome in the mouse and evidence for a genotype-specific effect of Russian tarragon in the regulation of insulin sensitivity

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Abstract

Published reports of botanical action are often hampered by the lack of generalized systematic approaches or by the failure to explore mechanisms that could confirm and extend the reported observations. Choice of mouse or rat housing conditions (singly or group housed) and imposed stress during handling procedures are often variable and can contribute significantly to differences in baseline phenotypes measured across studies. Differences can also be observed in the role of the extract in either the treatment of the metabolic syndrome or roles in the regulation of the emergence of metabolic syndrome. The choice of diet used can also vary between the different studies, and diet-botanical interactions must be considered. This minireview highlights the strategies being pursued by the Botanical Research Center Animal Research Core to evaluate the in vivo phenotypes of several botanical extracts during long-term feeding studies. We describe a phenotyping strategy that promotes a more rigorous interpretation of botanical action and can suggest or eliminate possible mechanisms that may be involved. We discuss the importance of selecting the mouse model, as background strain can significantly alter the underlying susceptibilities to the various components of metabolic syndrome. Finally, we present data suggesting that one of the major botanical extracts being studied, an extract of Russian tarragon, may manifest a mouse strain genotype–specific insulin-sensitizing phenotype.

1. Introduction

Obesity and type 2 diabetes mellitus are both components of a generalized disorder termed *metabolic syndrome* [1]. Accumulation of excess adipose tissue, a hallmark of obesity, leads to insulin resistance that precedes the development of type 2 diabetes mellitus. Other metabolic syndrome–associated comorbidities include dyslipidemia and hypertension. Individually, these components of metabolic syndrome are themselves considered as complex diseases, regulated by a poorly understood interaction of genetics, diet, and physical activity. The public health initiatives adopted in the United States and elsewhere have typically focused on reductions in food intake and increases in physical activity. The continued increase in the incidence of these diseases, however, in the developed countries, coupled with the emerging epidemic in developing nations and its increasing prevalence in children, suggests that other strategies are essential.

The identification and characterization of botanical extracts that can modulate the development of chronic diseases of obesity and type 2 diabetes mellitus are the major focus of the Botanical Research Center (BRC) based jointly at the Pennington Biomedical Research Center in Baton Rouge, LA, and Rutgers University, NJ. This minireview discusses the contribution of the Animal Research Core of the Botanical Center and the use of differing murine genetic models of metabolic syndrome to help define the mechanism of action of botanicals.

2. Rationale for the use of the mouse as a model for human metabolic syndrome

Despite more than 65 million years since the divergence of primate and rodent phylogenetic lineages, comparative analysis of the human and mouse genomes reveals that 99%

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of the genes associated with disease are evolutionarily conserved. Several attributes make the mouse an ideal experimental model for biomedical research: chronic diseases such as obesity, diabetes, cancer, autoimmunity, and muscular dystrophies seen in humans can and do occur to varying extents in different inbred mouse strains; they are easy and cost-effective to breed and maintain; a large number of genetically distinct inbred mouse strains exist; and the mouse genome is easily manipulated to generate transgenic and gene-deficient mutant mice. In addition, the genome of the commonly used C57BL/6J (B6) mouse has been completely sequenced (www.ensembl.org), making it easier to characterize the underlying genetic influences on phenotypic diversity.

3. Common mouse models for metabolic syndrome

Murine polygenic and monogenic models of the metabolic syndrome have been used to differing extents to characterize the effects of botanical extracts in the prevention and treatment of obesity and/or diabetes. Given that human obesity is predominantly polygenic in nature and overall genetic susceptibility can be modulated by energy-dense high-fat diets, the C57BL/6J (B6) high-fat dietary-induced obesity model is commonly used as a surrogate for the human disease. Male B6 mice, when fed a high-fat or high-fat + high-sucrose diet, rapidly develop obesity and manifest increased insulin resistance, hyperglycemia, dyslipidemia, and hypertension, all aspects of the metabolic syndrome [2,3]. The extent to which this mouse strain demonstrates a diabetes phenotype however is limited. The diabetes phenotype appears transient in nature and can resolve.

Several commonly used inbred mouse strains are resistant to high-fat dietary-induced obesity [4,5]. For example, the 129-related mouse strains (embryonic stem cells of which are commonly used in gene targeting experiments) do not demonstrate obesity when fed a high-fat diet. Mechanisms that regulate increased energy expenditure are more effectively induced in this mouse strain relative to the B6 inbred strain [6].

Another commonly used mouse obesity and diabetes model is the leptin-deficient obesity mouse mutant homozygous for the *lep*^{ob} allele. The leptin receptor (*lepr*^{db})–deficient mouse mutant also possesses a similar phenotype. Leptin is an important hormone, predominantly secreted by fat cells that interact with the leptin receptor isoforms expressed by neurons located in the hypothalamus. Changes in the level of circulating leptin alter feeding behavior, metabolism, and endocrine function [7]. Both male and female *lep*^{ob} mutants are obese and demonstrate hyperglycemia, glucose intolerance, elevated plasma insulin, subfertility, impaired wound healing, and increases in hormone production from both pituitary and adrenal glands. They are also hypometabolic and hypothermic, necessitating a higher temperature (>25°C) in the animal rooms to minimize thermogenic stress. It is quite common to see an incorrect use of murine genetic nomenclature in the botanical-related literature when describing leptin-deficient mice. Instead of being described as "B6-*ob* or *ob*/*ob* mice," the correct nomenclature of this mutant congenic strain when the mutation is on the B6 genetic background is C57BL/6.V-*lep*^{ob} (abbreviated as B6.V-*lep*^{ob}).

Genetic background plays an important role in regulating the diabetic phenotype in leptin-deficient mice. The B6.V-*lep*^{ob} strain, although demonstrating significant obesity, may not be the ideal choice to use in studies requiring significantly greater insulin resistance and type 2 diabetes mellitus. When the *lep*^{ob} mutation is homozygous on the closely related C57BL/Ks genetic background, the congenic C57BL/Ks.V-*lep*^{ob} mice are more severely diabetic, with regression of pancreatic islets and early adult lethality, than B6.V-*lep*^{ob} mutants [8]. B6.V-*lep*^{ob} mice live longer, in large part because they demonstrate only a transient hyperglycemia that resolves at around 14 to 15 weeks of age [9]. BALB/cJ congenic mice, when homozygous for the *lep*^{ob} mutation, are leaner than B6.V-*lep*^{ob} mutant mice and hence more fertile; but they demonstrate significantly greater diabetes [10]. Conversely, BTBR mice homozygous for *Lep*^{ob} are more obese than B6.V-*Lep*^{ob} mutant mice [11,12]. Thus, the same mutation on differing genetic backgrounds alters the degree of obesity, fertility, and diabetes. A potential drawback to the use of *Lep*^{ob} mutant mice as a model for metabolic syndrome is that the data generated must be interpreted in light of a leptin-deficient environment. Although leptin-deficient humans have been identified in the population, the incidence of this mutation is quite rare. Most human obesity and diabetes are attributable to a more complex polygenic origin.

The KK.Cg-*A*^{y} mutant mouse, also used as a model for metabolic syndrome, offers advantages over the use of the leptin-deficient mouse in that the development of obesity, insulin resistance, and diabetes in this model is polygenic and the mutant mice possess intact leptin and leptin receptor genes. The *A*^{y}, or yellow mutation, contains a 170–base pair deletion at the nonagouti (a) locus that extends into the upstream *Raly* gene [13]. As a result, expression of agouti is under the control of the *Raly* gene promoter. Thus, agouti is ectopically expressed in all tissues instead of its more limited expression profile in the skin. Cross talk between agouti and specific melanocortin receptors in the hypothalamus of *A*^{y}/+ heterozygous mice results in hyperphagia leading to an age-dependent metabolic syndrome phenotype [14-16]. Mice homozygous for *A*^{y}/*A*^{y} die in utero, presumably because of loss of *Raly* gene expression. Thus, genotyping of progeny derived from +/+ × *A*^{y}/+ mating schemes is not required; yellow-colored *A*^{y}/+ mice are easily distinguished from +/+ littermates. However, as found for different *Lep*^{ob} mouse mutant strains, genetic background effects can significantly modify the metabolic syndrome phenotype in *A*^{y}/+ mouse mutants.

Fig. 1 describes the effects of the *A*^{y} mutation when congenic on the B6 and KK inbred mouse strain genetic

backgrounds. B6.Cg-A^y/+ mice demonstrate a significant increase in body weight and adiposity, relative to the parental B6 mice, because of accumulation of fat mass that begins shortly after 7 weeks of age (Fig. 1, $t = 0$) and continues to increase for the next 3 months. In contrast, the parental KK/HlJ (KK) and congenic KK.Cg-A^y/+ mouse strains show no significant differences in body weight and adiposity throughout this same period. Measurements of circulating glucose and triglycerides suggest that KK.Cg-A^y/+ mice are significantly more diabetic and hypertriglyceridemic than the parental KK strain, but these phenotypes are not observed when the A^y mutation is congenic on the B6 genetic background. Thus, we conclude that the effect of the A^y/+ mutation is to confer increased adiposity on the B6 genetic background but increased diabetes (with corresponding insulin resistance) and hypertriglyceridemia on the KK genetic background. This phenotypic difference occurs despite the fact that the A^y mutation confers hyperphagia to both mouse strains, although again strain differences are apparent. A 20% increase in food intake is observed for B6.Cg-A^y/+ mice relative to B6, but a larger 45% increase is observed in KK.Cg-A^y/+ mice relative to KK.

Previous reports identify an extract derived from Russian tarragon (PMI-5011) as being an in vivo insulin sensitizer leading to significant reductions in fasting insulin in the treatment of the metabolic syndrome using the KK-A^y mouse model of type 2 diabetes mellitus [17]. Our earlier studies (data not shown) revealed that PMI-5011 did not have a significant effect on glucose, insulin, or adiposity in high-fat–fed obese B6 mice. Thus, to determine if there was a background strain–specific botanical effect, we compared the effectiveness of PMI-5011 treatment in 10-week–old B6.Cg-A^y/+ and KK.Cg-A^y/+ mutant male mice (Fig. 2). PMI-5011 feeding reduced circulating insulin concentrations in KK.Cg-A^y/+, but not in B6.Cg-A^y/+ mice. No effects on circulating glucose were noted in either of the 2 mouse models. Thus, this data suggest that the insulin-sensitizing effects of PMI-5011 are mouse background strain specific.

Genetic background effects on phenotypic traits are extraordinarily common in mouse genetic studies. It plays a significant role in determining the overall susceptibility of the individual to tumor susceptibility and regulates disease occurrence and progression, response to pharmaceuticals, and overall health status presumably because of allelic variation in functionally important modifier genes. These genes can influence phenotypes in subtle or profound ways; and the effects provide clues to the underlying pathways, networks, and systems that control biological traits [18,19]. Given the large degree of genetic variation observed in humans, understanding botanical-genotype interactions has

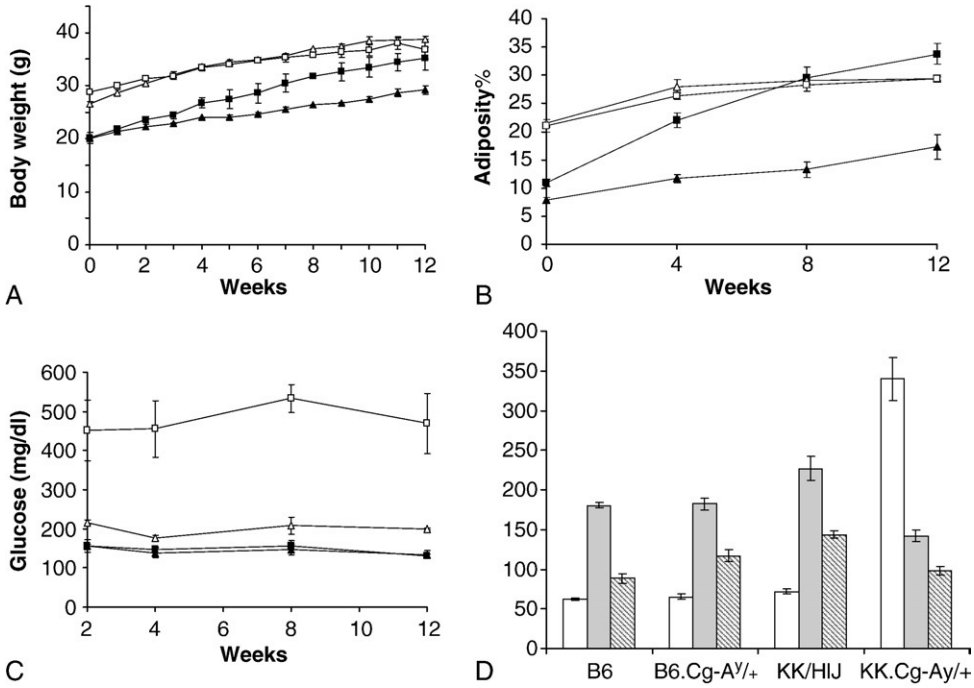

Fig. 1. Comparison of the effects of the A^y mutation when congenic on the KK and B6 genetic backgrounds. Male mice (n = 6) were purchased from Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age and fed a defined low-fat diet, D12329 (Research Diets), throughout the experiment. Symbols: B6, ▲; B6.Cg-A^y/+, ■; KK/HlJ (KK), Δ; KK.Cg-A^y/+, □. Time zero corresponds to mice having reached 7 weeks of age. Body weight (A) was measured weekly, and body composition (B) was measured monthly. No differences were observed between parental and mutant mouse strains in fat-free mass (not shown). Adiposity is calculated as mass of fat divided by body weight, expressed as a percentage. C, The circulating glucose concentrations measured in plasma recovered from the retroorbital sinus of mice that had been fasted for 4 hours (10:00 AM to 2:00 PM). D, A comparison of triglyceride (white bars), total cholesterol (gray bars), and high-density lipoprotein cholesterol concentrations (striped bars) in the 4 mouse strains, separately measured in 15 μL of whole blood using a Cardiochek Bioscanner 2000 (Polymer Technology Systems, Inc, Indianapolis, IN) in 4-hour–fasted mice taken after completion of the 12 weeks of phenotypic monitoring.

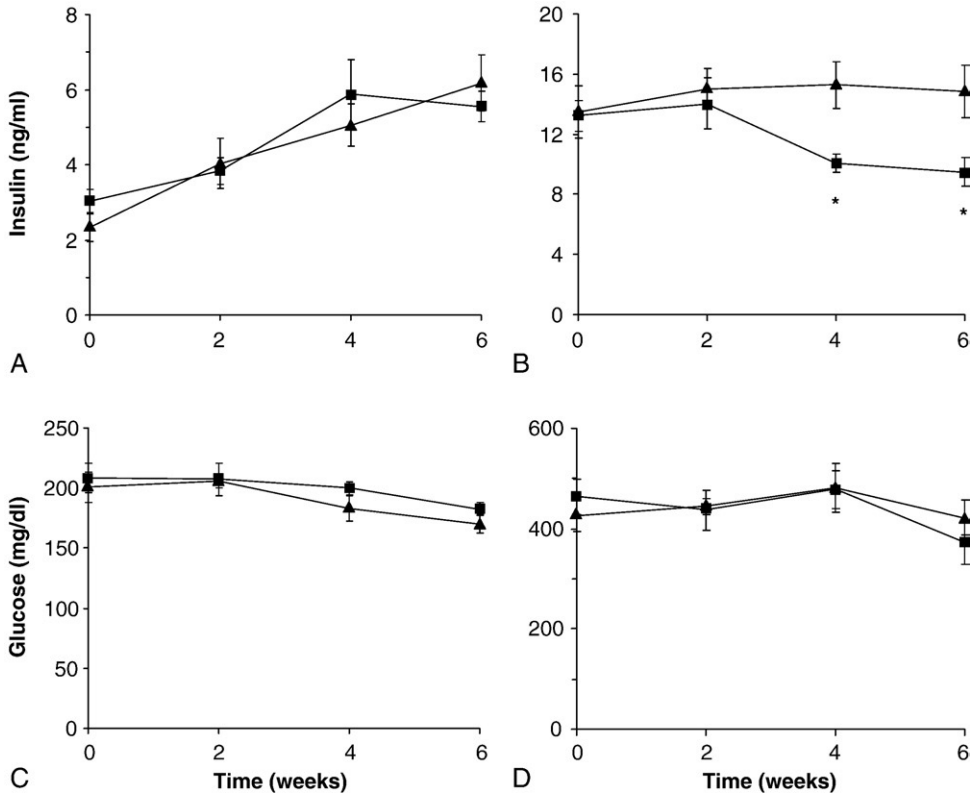

Fig. 2. Differential effects of PMI-5011, an extract from Russian tarragon, on plasma insulin and glucose concentrations in B6.Cg-A^y/+ (A and C, respectively) and KK.Cg-A^y/+ (B and D, respectively) mutant male mice. Eighteen mice (purchased from Jackson Laboratory) were used for each of the 2 mouse strains, split into 2 groups of 9 each. One group was fed PMI-5011 (■) that had been incorporated into a defined low-fat diet (D12329, Research Diets) at a concentration of 1% (wt/wt). The control group was fed the same low-fat diet without PMI-5011 (▲). Time zero corresponds to mice that were 10 weeks of age, and all mice had been fed the defined low-fat diet for 4 weeks. Plasma was collected from the retroorbital sinus at the indicated time points from mice that had been fasted for 4 hours (10:00 AM to 2:00 PM). No effects of PMI-5011 were observed on food intake, body weight, adiposity, fat mass, and fat-free mass in either of the 2 mouse models (not shown).

clear and important health considerations. Inbred mouse strains represent a valuable resource to exploit genetic studies in which allelic variants of important genes can be mapped and ultimately identified.

4. Dietary considerations—the advantages of defined diets over chow

Plant extracts contain a large and complex array of phytochemicals. Thus, the in vivo phenotypic profiles seen in animals fed a diet supplemented with a botanical extract are complicated not only by the possibility of interactions between different phytochemicals present in the same extract but also between one or more phytochemicals and chemical components present in the base diet. In addition, it is well established that dietary macronutrient composition plays a major role in the development of the metabolic syndrome [20-23]. Even for each class of macronutrient, there are differences in the source (animal vs plant) and chemical composition of proteins, carbohydrates (simple vs complex), and fats (unsaturated, monosaturated, *cis*- and *trans*-saturated). Undefined chow diets typically contain variable levels of daidzen, genistein, and phytoestrogens [24]. Often, soymeal, alfalfa, and cornmeal are used as dietary supplements; and animal fat is included in some formulations. For these reasons, the BRC uses chemically defined and purified low-fat and high-fat diets for all feeding studies. Standardization of diet has a major advantage in that it promotes a direct comparison of the in vivo effects of different botanicals and purified phytochemicals. The actual defined low-fat diets used by the BRC are D12329 (11% kcal fat; Research Diets, New Brunswick, NJ), used in studies using the spontaneously diabetic and insulin-resistant KK.Cg-A^y/+ mouse, and a matched high-fat and high-sucrose diet, D12331 (58% kcal fat, Research Diets), used for dietary-induced obesity models.

5. Metabolic profiling

Dietary studies involving the use of botanical extracts can take several forms. Feeding young and lean B6 mice with test extracts in the context of a high-fat diet provides information concerning the effectiveness of the extract in regulating weight gain induced by a high-fat diet. However,

first making the mice obese by 3 months of high-fat–diet feeding before initiating feeding with extracts provides useful information on the role of the extract in the treatment of metabolic syndrome phenotypes. It is useful to consider both dietary models to evaluate the same extract in the prevention and treatment of disease. Significant challenges remain in the evaluation of the role of botanical supplements in regulating metabolic syndrome. The concentration of the botanical extract in the diet can be quite variable. Typically, incorporation of the test extract at concentrations greater than 2% to 5% can potentially lead to calorie dilution effects on the base diet, promoting reduced weight gain or increased weight loss depending upon the feeding paradigm being used. We typically measure body composition using nuclear magnetic resonance spectroscopy on live, unanesthetized mice at monthly intervals during these feeding studies (typically 8-12 weeks in duration) using a Bruker mq10 minispec (Bruker Optics Inc, The Woodlands, TX). This approach controls for the possibility of significant changes in body composition occurring in the absence of significant changes in body weight. If nuclear magnetic resonance spectroscopy is not available, then direct measurements of fat pad weights at necropsy can be a suitable surrogate for estimating adiposity.

If changes in body weight or body composition are observed, it is essential that this phenotype be correlated with measurements of food intake conducted in the same mice model before these differences become significantly different. Food intake is measured over a period of 1 week on at least 2 different occasions during the feeding study. It is important to take spillage into account, and this may be significant. Although food intake can be measured in group-housed situations, greater power can be obtained if the animals are singly housed. In this case, feed efficiency (the amount of food eaten for a given increase in body weight) can be measured per mouse. If food intake is unchanged between the treatment and control groups AND effects on body weight or body composition are observed, then these suggest that changes in energy expenditure and/or physical activity and/or thermogenesis may be modulated by the extract being tested. The 2 former phenotypes can be explored using indirect calorimetry. The test animals need to be singly housed and enough time should be allowed to pass for adaptation to the smaller metabolic phenotyping chambers (typically 3-5 days) before reliable measurements can be obtained. Indirect calorimetry using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH) allows for simultaneous measurements of respiratory exchange ratio, oxygen consumption, carbon dioxide production, physical activity (using light beam splitting), and food intake [25].

If reductions in food intake are observed, then other mechanisms may be suggested. At its simplest, the botanical extract–based diet combination may not be palatable at the concentrations used. Reductions in extract concentrations may resolve this issue. Alternatively, the mice may demonstrate taste aversion or other gastric distress upon consumption or metabolism of the dietary supplement. In this case, a "conditioned taste avoidance" test can be performed [26-28]. By pairing the feeding with the botanical extract with the presentation of saccharin, a sugar normally preferred by mice over water alone, an association between the 2 separate items is generated. The drinking response to saccharin is then tested after removal of the test diet. A reduction in relative saccharin intake relative to control diet–fed mice would demonstrate a conditioned taste avoidance. Reductions in food intake could also be associated with decreased gastric emptying rates. Measurements of stomach contents at several intervals after presentation of dietary-supplemented diet and control diet to overnight food-deprived mice could be used to test this possibility.

It is possible that some botanical extracts may alter normal thermogenesis. The use of biotelemetry offers unique advantages in the measurements of core body temperature throughout the day and night cycle of test animals. Our facilities use surgically implanted transponders (Mini-Mitter, Bend, OR) that are suitable for long-term (12-week) studies. Core body temperature and physical activity of mice in their normal home cages are continuously monitored at 2-minute intervals.

Measurements of the glucose-insulin axis of mice require periodic blood sampling. Glucose concentrations in most murine models can be reliably measured using one of the many types of glucometers available for blood glucose monitoring in diabetic patients. Glucose levels in the whole blood of diabetic KK.Cg-A^y/+ mice, however, can easily exceed 600 mg/dL, beyond the upper calibrated range for many of these instruments. Thus, other methods must be used to measure glucose in plasma or sera. If changes in glucose and/or insulin concentrations are observed, it is often useful to address the functional significance of these changes by performing glucose and insulin tolerance tests. Ultimately, using the services of the National Institutes of Health–supported mouse metabolic phenotyping centers (www.mmpc.org) may be required to perform hyperinsulinemic-euglycemic clamps and thereby obtain useful information on rates of gluconeogenesis and whole-body and tissue-specific glucose disposal.

A goal of the BRC is to identify key signaling pathways in metabolic syndrome that are modified by botanical action. As candidate genes and pathways are identified, it will be necessary to obtain in vivo evidence supporting these observations. The Animal Research Core will select the most appropriate mouse mutants to test in functional assays with the appropriate botanical extract or purified phytochemical. The mouse is an excellent model organism because of the availability of specific mutants that are deficient or otherwise modified in the expression of many genes and proteins. Phenotyping the effects of botanicals in these mutants, many of which are commercially available, allows for the verification of hypotheses generated from other molecular and biochemical approaches.

Acknowledgment/Conflict of Interest

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References

[1] Teran-Garcia M, Bouchard C. Genetics of the metabolic syndrome. Appl Physiol Nutr Metab 2007;32:89-114.
[2] Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN. Diet-induced type II diabetes in C57BL/6J mice. Diabetes 1988;37:1163-7.
[3] Petro AE, Cotter J, Cooper DA, Peters JC, Surwit SJ, Surwit RS. Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse. Metabolism 2004;53:454-7.
[4] West DB, Boozer CN, Moody DL, Atkinson RL. Dietary obesity in nine inbred mouse strains. Am J Physiol 1992;262:R1025-32.
[5] West DB, Waguespack J, McCollister S. Dietary obesity in the mouse: interaction of strain with diet composition. Am J Physiol 1995;268: R658-65.
[6] Almind K, Kahn CR. Genetic determinants of energy expenditure and insulin resistance in diet-induced obesity in mice. Diabetes 2004;53: 3274-85.
[7] Badman MK, Flier JS. The adipocyte as an active participant in energy balance and metabolism. Gastroenterology 2007;132:2103-15.
[8] Coleman DL, Hummel KP. The influence of genetic background on the expression of the obese (Ob) gene in the mouse. Diabetologia 1973;9: 287-93.
[9] Coleman DL, Schwizer RW, Leiter EH. Effect of genetic background on the therapeutic effects of dehydroepiandrosterone (DHEA) in diabetes-obesity mutants and in aged normal mice. Diabetes 1984;33:26-32.
[10] Qiu J, Ogus S, Mounzih K, Ewart-Toland A, Chehab FF. Leptin-deficient mice backcrossed to the BALB/cJ genetic background have reduced adiposity, enhanced fertility, normal body temperature, and severe diabetes. Endocrinology 2001;142:3421-5.
[11] Clee SM, Nadler ST, Attie AD. Genetic and genomic studies of the BTBR ob/ob mouse model of type 2 diabetes. Am J Ther 2005;12: 491-8.
[12] Stoehr JP, Byers JE, Clee SM, Lan H, Boronenkov IV, Schueler KL, et al. Identification of major quantitative trait loci controlling body weight variation in ob/ob mice. Diabetes 2004;53:245-9.
[13] Michaud EJ, Bultman SJ, Klebig ML, van Vugt MJ, Stubbs LJ, Russell LB, et al. A molecular model for the genetic and phenotypic characteristics of the mouse lethal yellow (Ay) mutation. Proc Natl Acad Sci U S A 1994;91:2562-6.
[14] Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, et al. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 1994;371:799-802.
[15] Ollmann MM, Lamoreux ML, Wilson BD, Barsh GS. Interaction of agouti protein with the melanocortin 1 receptor in vitro and in vivo. Genes Dev 1998;12:316-30.
[16] Stark KL. Agrp, a novel gene implicated in the control of feeding. Expert Opin Investig Drugs 1998;7:859-64.
[17] Ribnicky DM, Poulev A, Watford M, Cefalu WT, Raskin I. Antihyperglycemic activity of Tarralin, an ethanolic extract of *Artemisia dracunculus* L. Phytomedicine 2006;13:550-7.
[18] Nadeau JH. Modifier genes and protective alleles in humans and mice. Curr Opin Genet Dev 2003;13:290-5.
[19] Nadeau JH. Modifier genes in mice and humans. Nat Rev Genet 2001; 2:165-74.
[20] Tremblay A. Nutritional determinants of the insulin resistance syndrome. Int J Obes Relat Metab Disord 1995;19(Suppl 1):S60-8.
[21] Reed DR, Bachmanov AA, Beauchamp GK, Tordoff MG, Price RA. Heritable variation in food preferences and their contribution to obesity. Behav Genet 1997;27:373-87.
[22] Bachmanov AA, Reed DR, Tordoff MG, Price RA, Beauchamp GK. Nutrient preference and diet-induced adiposity in C57BL/6ByJ and 129P3/J mice. Physiol Behav 2001;72:603-13.
[23] Brunner EJ, Wunsch H, Marmot MG. What is an optimal diet? Relationship of macronutrient intake to obesity, glucose tolerance, lipoprotein cholesterol levels and the metabolic syndrome in the Whitehall II study. Int J Obes Relat Metab Disord 2001;25:45-53.
[24] Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, et al. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. Lab Anim Sci 1999;49:530-6.
[25] Perseghin G. Pathogenesis of obesity and diabetes mellitus: insights provided by indirect calorimetry in humans. Acta Diabetol 2001;38: 7-21.
[26] Parker LA. Taste avoidance and taste aversion: evidence for two different processes. Learn Behav 2003;31:165-72.
[27] Kallman MJ, Lynch MR, Landauer MR. Taste aversions to several halogenated hydrocarbons. Neurobehav Toxicol Teratol 1983;5:23-7.
[28] Springer AD, Fraley SM. Extinction of a conditioned taste aversion in young, mid-aged, and aged C57/BL6 mice. Behav Neural Biol 1981; 32:282-94.