Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet

RÉMY BURCELIN, VALÉRIE CRIVELLI, ANABELA DACOSTA, ALEXANDRA ROY-TIRELLI, AND BERNARD THORENS

Institute of Pharmacology and Toxicology, Lausanne University, 1005 Lausanne, Switzerland

Published 23 July 2001; accepted in final form 25 November 2001

CONTROL OF BODY WEIGHT requires balance between food intake and energy expenditure. It is now well recognized that maintaining this balance depends on very intricate regulatory mechanisms, in great part controlled by hypothalamic circuits that integrate signals received from the periphery. These are indicators of the body energy state and include the adipocyte hormone leptin but also insulin and variations in blood glucose concentrations. Neurons sensitive to these hormones and to glucose are located, in particular, in the arcuate nucleus and express the hypothalamic hormones neuropeptide Y, agouti gene-related protein (AGRP), proopiomelanocortin [giving rise to the active α-melanocyte-stimulating hormone (α-MSH) peptide], and cocaine and amphetamine-regulated transcript (7). Through activation of second-order neurons and the autonomic nervous system, energy homeostasis is maintained by the adaptive regulation of diverse physiological functions such as glucose utilization in peripheral tissues, hormone secretion, food intake, and energy expenditure.

Imbalance in this homeostatic process can be caused by mutations in single “obesity” genes like the leptin or leptin receptor genes (25, 34). However, in humans, these monogenic forms of obesity are extremely rare (4, 17). More common are the association of mutations in some hypothalamic receptors, such as the melanocortin receptor MC4R (5, 29, 32), which is activated by α-MSH or inhibited by AGRP, with an increased susceptibility to obesity development. In most cases of obesity, however, even though there is a well recognized genetic component in the development of this disease, the genes involved are not known. It is also well established that environmental factors combine with the susceptibility genes to induce obesity. In particular, the lack of physical exercise and high calorie-containing diets are major parameters favoring the development of obesity and diabetes. The importance of the environmental factors in inducing these diseases is exemplified by the study of twins who were discordant for non-insulin-dependent diabetes mellitus (19, 27, 28). It was shown that lipid oxidation was increased, whereas glucose oxidation was reduced, in the diabetic compared with the nondiabetic twins. In rats and mice, high-fat feeding induces obesity and diabetes (1, 18, 20, 24, 33). Interestingly, however, it was observed that, in C57BL/6J mice fed a high-fat diet for several weeks, development of obesity and diabetes was not uniform, and different lean and obese phenotypes could be observed. In this particular strain, vari-
ations in genetic background cannot be proposed as an explanation for the differences in body weights.

Therefore, we hypothesized that the lean or obese phenotypes are due to different regulatory mechanisms, resulting from a specific adaptation of each individual to the nutritional stress. The aim of this study was thus to characterize glucose metabolism in C57BL/6J mice, which become, after a long period of high-fat feeding, obese diabetic (ObD), lean diabetic (LD), or lean nondiabetic (LnD). We report that high-fat, carbohydrate-free diet (HFD)-fed mice were insulin resistant in vivo. However, in the fasting state, muscle glucose clearance was reduced, normal, or higher than normal in the ObD, LD, and LnD mice, respectively. These differences were not observed in vitro in isolated muscles, suggesting that the regulatory mechanism was dependent on an in vivo control.

MATERIAL AND METHODS

Animals and Reagents

Four- to five-week-old male C57Bl/6J mice (IFFACREDO, L’Arbresle, France) were fed a normal chow diet (NC, energy content: 12% fat, 28% protein, and 60% carbohydrate) or an HFD diet (energy content: 72% fat (corn oil and lard), 28% protein, and <1% carbohydrate), as described (2). Eight to ten mice per cage were kept on an 8 AM-to-8 PM dark cycle. The mice were allowed to recover for 4–6 days. The day before the experiments and blood sampling, food was removed at 8 AM, and the mice were kept in a clean new cage for 6 h.

Intraperitoneal Glucose Tolerance Test

An intraperitoneal injection of glucose [intraperitoneal glucose tolerance test (iPGTT), 1 g/kg body wt] was performed in 6-h-fasted mice after 3, 6, and 9 mo of diet treatment. Blood glucose levels were monitored from the tip of the tail vein with a glucose meter (Roche Diagnostic, Rotkreuze, Switzerland) from a 3.5-μl sample of tail blood at 0, 5, 10, 15, 20, 25, 30, 45, and 60 min after glucose injection.

In Vivo Glucose Utilization Rate

To determine the rate of glucose utilization an indwelling catheter was placed into the femoral vein under anesthesia, sealed under the back skin, and glued onto the top of the skull. The mice were allowed to recover for 4–6 days. The day of the experiment, the mice were fasted for 6 h. The whole body glucose utilization rate was determined in basal and in hyperinsulinemic eucligenic conditions. In the basal state, high-performance liquid chromatography-purified D-[3-3H]glucose (NEN Life Science, Boston, MA) was continuously infused through the femoral vein at a rate of 10 μCi·kg⁻¹·min⁻¹ for 3 h. Under hyperinsulinemic conditions, insulin infusion was increased at a rate of 18 μU·kg⁻¹·min⁻¹ for 3 h, but D-[3-3H]glucose was infused at a rate of 30 μCi·kg⁻¹·min⁻¹, higher than in the basal condition, to ensure a detectable plasma D-[3-3H]glucose enrichment. Throughout the infusion, blood glucose was assessed with a blood glucose meter from blood samples (3.5 μl) collected from the tip of the tail vein when needed. Euglycemia was maintained by periodically adjusting a variable infusion of 16.5% glucose. Plasma glucose concentrations and D-[3-3H]glucose specific activity were determined in 5 μl of blood sampled from the tip of the tail vein every 10 min during the last hour of the infusion.

In Vivo Glucose Utilization Index in Individual Tissues

To determine an index for the individual tissue glucose utilization rate a flash injection of 30 μCi per mouse of D-2-[3H]deoxyglucose (D-[3H]2-DG; NEN Life Science) through the femoral vein was performed 60 min before the end of the infusions (3). Plasma D-[3H]2-DG disappearance and glucose concentration were determined in 5-μl drops of blood sampled from the tip of the tail vein at 0, 5, 10, 15, 20, 25, 30, 45, and 60 min after the injection.

In Vitro Individual Muscle Glucose Utilization

After cervical dislocation, the hemidiaphragm, the extensor digitorum longus (EDL), and the soleus muscles were rapidly isolated, tied separately by silk threads to the tendons or the diaphragm hilus, and immersed for 20 min into an incubation medium [Krebs-Ringer bicarbonate (pH 7.3) supplemented with 1% bovine serum albumin (fraction V, pH 7.0; Sigma, St. Louis, MO) and 2 mM sodium pyruvate], as described (22). Under an atmosphere containing 5% CO2 and 95% O2, the muscles were then incubated in the medium with or without 10 nM insulin for 60 min at 37°C. Thereafter, the muscles were immersed for 20 min in the incubation medium supplemented with D-[3H]2-DG (0.1 mM, 0.5 μCi/ml). During this immersion the D-[3H]2-DG is metabolized and accumulates as D-2-[3H]deoxyglucose 6-phosphate. To stop the reaction, the muscles were immersed in ice-cold saline buffer, washed for 30 min, and then dissolved in 1 M NaOH at 55°C for 60 min. An aliquot of the extract was neutralized with 1 M HCl and spun down, and the 3H-labeled radioactivity was counted in the presence of a scintillation buffer. Sample aliquots were used for protein determination.

Analytical Procedures and Determinations

Blood parameters. Plasma glucose concentration was determined by a glucose oxidase method (Trinder Kit; Sigma Diagnostic, St. Louis, MO). Plasma insulin concentration was determined by ELISA (Mercodia, Uppsala, Sweden), from 10 μl of plasma sampled from the tail vein during the iPGTTs or radioimmunoassays in all others instances (Linco, St. Charles, MO). Plasma FFA were determined by an enzymatic colorimetric reaction using the acyl-CoA synthase and acyl-CoA dehydrogenase enzymes (NEFA C; Wako, Neuss, Germany).

Isotope Measurements. For glucose turnover measurements D-[3-3H]glucose enrichments were determined from total blood after deproteinization by a Zn(OH)₂ precipitate as described (3, 21). Briefly, an aliquot of the supernatant was evaporated to dryness to determine the radioactivity corresponding to D-3-3H. In a second aliquot of the same supernatant, glucose concentration was assessed by the glucose oxidase method.

For individual tissue glucose uptake measurements, plasma D-[3H]2-DG was determined from total blood after deproteinization with a Zn(OH)₂ precipitate as described (3, 21). Tissue D-[3H]2-DG and D-2-[3H]deoxyglucose 6-phosphate content were determined as previously described (3, 21). Briefly, a piece of each tissue was dissolved in 1 M NaOH at 55°C for 60–120 min and then neutralized with 1 M HCl. D-2-[3H]deoxyglucose 6-phosphate and D-[3H]2-DG were differentially precipitated by the use of a zinc hydroxide (0.3 M) solution or a perchloric acid solution (6%) (3).

Calculations

Calculations for glucose turnover measurements were made from parameters obtained during the last 60 min of the
infusions in steady-state condition as described (3, 21). Briefly, the d-[3-3H]glucose specific activity was calculated by dividing the d-[3-3H]glucose enrichment by the plasma glucose concentration. The whole body glucose turnover rate was calculated by dividing the rate of d-[3-3H]glucose by the d-[3-3H]glucose plasma specific activity. Because the mice had different fasted glycemias, we calculated the whole body glucose clearance rates by dividing the glucose turnover rate by the glycemic level. For each mouse, the mean values were calculated and averaged with values from mice of the same group. Mice showing variations of the steady-state d-[3-3H]glucose specific activity by 15% during this time period were excluded from the study.

Statistical Analysis

Results are presented as means ± SE or SD, as specified in figures. Statistical significance of differences was analyzed by using Student's t-test for unpaired, bilaterally distributed values of equal variance. Values were considered different when P < 0.05.

RESULTS

Body Weight Gain

One-month-old male C57BL/6J mice were fed a NC or a HFD diet for 9 mo. The body weights of the HFD mice were similar to those of the NC mice for the first 3 mo (Fig. 1A). After three more months of HFD feeding, the body weights further increased by 25%, whereas the body weights of the NC-fed mice increased only by 11%. Strikingly, the body weights of the HFD mice were widely scattered at 6 and 9 mo, with values lower, similar, or higher than the body weights of the NC-fed mice.

Fasted Blood Glucose and Insulin Levels

Fasted blood glucose levels were measured in 6-h-fasted mice (Fig. 1B). Fasted glycemias remained steady at ~5.5 mM in NC-fed mice during the entire study. Conversely, after 3 mo of HFD, the means of the fasted glycemias were higher than those of the NC mice and remained so until the end of the study. In addition, the glycemias of the HFD mice were highly scattered.

Fasted plasma insulin levels were measured at 3, 6, and 9 mo of dietary treatment. The mean plasma insulin levels were higher in the HFD mice than in the NC mice at all time points studied (Fig. 1C). Importantly, these values were also highly scattered after 9 mo of HFD.

IPGTT

IPGTTs were performed in 6-h-fasted mice, and the area under the curves (AUCs) of the glycemias were calculated at 3, 6, and 9 mo of HFD or NC (Fig. 1D). In the NC mice, the AUCs remained steady throughout the study. After 3 mo of HFD, the AUCs were already higher than those of the NC mice and remained elevated after 6 and 9 mo.

Classification and Distribution of HFD Mice in Diabetic and Obese Subgroups

Because body weights and glucose AUCs of the HFD mice were widely scattered, we grouped the mice according to their obese and diabetic phenotypes. The glucose AUCs were therefore plotted against the body weight measured for each individual NC and HFD
mouse at 3, 6, and 9 mo of diet (Fig. 2). HFD mice were considered obese or lean when their body weight was higher than the mean $+ 3 \text{ SD}$ or lower than the mean $- 3 \text{ SD}$ of the NC mice, respectively. The other mice were considered as having an intermediate phenotype and were not further studied. Similarly, HFD mice were considered diabetic or nondiabetic when their glucose AUC was higher than the mean $+ 3 \text{ SD}$ or lower than the mean $- 3 \text{ SD}$ of the NC mice, respectively.

On the basis of these criteria, the HFD mice could be classified in four groups: the lean nondiabetic (LnD), the lean diabetic (LD), the obese diabetic (ObD), and the intermediate phenotype (Int). The definition of each group has been detailed in RESULTS. The percentiles of each population are represented in each corresponding rectangles.

Interestingly, when the body weight was plotted against the fasted blood glucose concentration for each individual mouse, the distribution of the phenotypes was similar to the described correlation (not shown).

Glucose, Insulin, Glucagon, FFA, and Leptin Levels in HFD Mouse Subgroups

After 9 mo of HFD, only the ObD mice were hyperinsulinemic in the fasted state (Fig. 3A). Conversely, the lean mice were slightly hypoinsulinemic, but the difference with the controls did not reach statistical significance. Plasma glucose levels were higher in the ObD and LD mice than in the NC and LnD mice (Fig. 3B). The insulin-to-glucose ratio of the obese were higher than those of the lean and NC mice (Fig. 3C), and the insulin-to-glucose ratios were lower in the lean than in the obese mice. When measured at 30 min after initiation of an IPGTT, the ObD mice were frankly hyperinsulinemic ($231 \pm 21 \text{ U/ml}$) compared with NC mice ($77 \pm 7 \text{ U/ml}$), and the LD and LnD mice were also hyperinsulinemic in these conditions ($155 \pm 9$ and $110 \pm 8 \text{ U/ml}$, respectively) but less than the ObD mice.
Fasted plasma glucagon level was higher in the LD group (97 ± 14 ng/ml) than in the NC mice (65 ± 4 ng/ml). No differences were detected among the ObD, LnD (74 ± 5 and 61 ± 7 ng/ml, respectively), and NC mice. Fasted FFA levels were similar between all groups of mice (0.942 ± 0.212, 0.907 ± 0.185, 0.955 ± 0.056, 0.902 ± 0.05 mM in NC, LD, LnD, and ObD mice, respectively). However, in the fed state, the FFA levels were higher in all HFD mice (1.235 ± 0.107, 1.618 ± 0.079, and 1.162 ± 0.082 mM in NC, LD, LnD, and ObD mice, respectively) than in the NC mice (0.488 ± 0.015 mM, P < 0.05). Plasma leptin levels were measured in fed mice and were: NC mice, 5.8 ± 1.8 ng/l (n = 6); LnD mice, 14.38 ± 2.6 ng/ml (n = 7); LD mice, 18.3 ± 1.24 ng/ml (n = 8); ObD mice, 44.81 ± 2.99 ng/ml (n = 36).

Analysis of Insulin Resistance and Glucose Turnover

Insulin sensitivity was assessed by the euglycemic hyperinsulinemic clamp technique after 9 mo of HFD (Fig. 4). Insulin action was reduced in all HFD mice by 60, 40, and 32% in the ObD, LD, and LnD mice, respectively, compared with NC mice, indicating marked insulin resistance. However, the degree of resistance was significantly higher in ObD compared with LD and LnD mice.

We then assessed glucose turnover (GTO) in vivo in 6-h-fasted HFD and NC mice and calculated glucose clearance, because the blood glucose levels were different between the mice. The glucose clearance rate was reduced by 60% in ObD mice compared with the NC mice (Fig. 5). Conversely, compared with NC mice, glucose clearance was similar in the LD or increased by 40% in the LnD mice.

In Vivo and In Vitro Individual Tissue Glucose Utilization Rate

The rate of individual tissue glucose clearance was then measured in 6-h-fasted HFD and NC mice. Compared with NC mice, the glucose clearance rate of the ObD mice was increased in white and brown adipose tissue, was similar in the diaphragm, soleus, and heart, and was reduced in the EDL and the whole hindlimb muscles (Fig. 6). In the LD mice, the glucose clearance rate was higher for the diaphragm, soleus, heart, and brown adipose tissue but lower for the EDL.
and total hindlimb muscles. In the LnD mice, the glucose clearance rate was increased in the diaphragm, soleus, EDL, and total hindlimb muscles and heart and brown adipose tissue. The glucose clearance of the white adipose tissue was similar between NC mice and the lean mice.

To determine whether the different glucose clearance rates observed in the soleus, diaphragm, and EDL muscles among the four groups of mice were constitutive or dependent on an in vivo regulatory mechanism, we analyzed in vitro glucose utilization in these muscles. Glucose utilization in the presence and absence of insulin was similar in the muscles isolated from the different groups of mice studied (Fig. 7A). The only difference was a reduced insulin-stimulated glucose transport activity in the diaphragm of the LnD mice.

**GLUT4 mRNA Concentration**

To determine whether the differences in muscle glucose utilization among all groups of mice were related to variations in GLUT4 expression, we quantified GLUT4 mRNA in the hindlimb (Fig. 7B). No differences were noted among all groups of mice.

**DISCUSSION**

Here, we report that C57BL/6J mice fed an high-fat diet for 9 mo display heterogenous metabolic adaptation. Three well-defined phenotypes can be distinguished: ObD (47% of the mice), LD (12%), and LnD (12%), the rest of the mouse population presenting intermediate phenotypes. Importantly, whatever their phenotype, all of the HFD mice became insulin resistant but displayed very variable glucose clearance rates. Compared with NC mice, these rates were markedly reduced in ObD mice, whereas they were similar or higher in LD and LnD mice, respectively. In vivo, the elevated glucose clearance rate of the LnD mice was associated with higher glucose utilization by all of the muscles analyzed. In vitro, glucose utilization rates were, however, similar in muscles isolated from control and LnD mice. This indicates that the in vivo increased glucose clearance may be controlled by factor(s) distinct from insulin.

The mean body weight of a population of C57BL/6J mice fed an HFD diet progressively increased over that of mice fed an NC diet, and this became evident between the 3rd and the 6th mo of dietary treatment. Diabetes, as defined by fasted hyperglycemia and glucose intolerance, appeared earlier. In particular, glucose intolerance was detected in 40% of the HFD mice after 3 mo, at a time when body weights were still in the normal range. Thus obesity became apparent after establishment of the diabetic phenotype. This unexpected timing of diabetes/obesity development could be due to the absence of carbohydrates in the diet used in the present study. Indeed, in separate experiments where mice were fed a high-fat, high-carbohydrate diet for 9 mo, they became obese and diabetic after the 3rd mo of treatment (not shown).

A major result of our study was that the mice that remained lean and nondiabetic (LnD) had a rate of glucose clearance that was significantly higher than that of the NC mice. In contrast, the glucose clearance rate of the ObD mice was lower than in control mice. The LD mice represented a still different phenotype, since their rate of glucose clearance was similar to that of the NC mice and their insulinemia was similar to that of the LnD mice. This, therefore, indicates that, at some point during HFD feeding, the different subgroups of mice underwent different metabolic adaptation. Interestingly, there is a good correlation between the capacity to increase above, or at least maintain glucose clearance rates at the level of, the NC mice and

**Fig. 6. Rates of individual tissue glucose clearance after 9 mo of HFD or NC feeding.** d-2-[3H]deoxyglucose was injected to determine in vivo the glucose clearance rate (μl/mg -1·min -1) by gonadal white adipose tissue (WAT), liver, and skin (A), and by the diaphragm (Dia), soleus muscle (Sol), extensor digitorum longus muscle (EDL), total hindlimb (HL), heart, and interscapular brown adipose tissue (BAT) (B) in the fasted state in NC (filled bars) and HFD 9-mo-fed ObD (open bars), LD (hatched bars), LnD (grey bars) mice. Five to six mice per group were studied. *Statistically different from NC-fed mice when P < 0.05.
the resistance to gain body weight. Importantly, this happened even though insulin resistance was present and insulin levels were not increased. This suggests that adaptation was due to some other factor. Analysis in vivo and in vitro of the rates of glucose utilization in individual tissues revealed that this factor was not intrinsic to muscles studied but was dependent on the in situ environment.

What can be the in vivo factors leading to increased glucose uptake? FFA concentrations in the fasted state were similar between all HFD and NC mice and could not explain insulin resistance or increased glucose clearance capacity. Leptin can increase muscle glucose utilization in the fasted state (8, 15). However, in the HFD mice, the leptin concentration was proportional to the body weight. Leptin levels were indeed elevated in obese mice, but whole body glucose utilization was low, suggesting that leptin could not be responsible for this phenotype. However, we cannot rule out that the obese mice were leptin resistant whereas the lean mice were not. Consequently, because the lean mice were characterized by higher-than-normal leptin levels, the hormone could be associated with the increased glucose utilization rate. It is noteworthy that the leptin levels were higher in the HFD than in the NC mice. Although the excessive leptin levels of the ObD mice could be directly related to the excessive fat mass, this was not the case for the lean mice. For the latter, the excessive leptin levels could be due to a gene regulation mechanism. Tumor necrosis factor-α (TNF-α) (6), resistin (23), or liver-dependent factors (9, 14) have been proposed to control muscle glucose utilization by acting directly as endocrine/paracrine factors (6, 9, 14). Although TNF-α and resistin could induce the observed insulin resistance, other adipocytes or liver factors may be involved in the increased muscle glucose clearance in the lean HFD mice. Finally, it has been suggested that the HFD-resistant and -sensitive phenotypes could be related to variations of the sympathetic activity and neural glucose sensitivity (11–13, 31). The role of the ANS in the regulation of muscle glucose utilization has been shown in other instances (3, 9, 15, 16, 30).

Fig. 7. Rates of individual tissue glucose uptake in vitro in isolated muscles after 9 mo of HFD or NC feeding and muscle GLUT4 mRNA concentration. A: glucose utilization was assessed in soleus (left), diaphragm (middle), and EDL (right) muscles in NC- and HFD-fed mice in the fasted state in NC (filled bars) and HFD 9-mo-fed ObD (open bars), LD (hatched bars), and LnD (grey bars) mice. The mean ± SE glucose utilization rate in the presence and absence of insulin are represented. Five to six mice per group were studied. *Statistically different from NC-fed mice when $P < 0.05$. B: Northern blot analysis of muscle GLUT4 mRNA. A representative Northern blot is shown, where no differences were noted between groups.
One other possibility to explain the increased rate of glucose uptake in muscles could be the higher expression of GLUT4 in muscles fibers. Indeed, overexpressing the glucose transporter GLUT4 specifically in the skeletal muscles increases glucose transport activity (10, 26). However, we did not detect any differences between the concentration of GLUT4 mRNA in hindlimbs of the three groups of HFD mice vs. the NC mice (not shown), and no increase in glucose transport activity was observed in vitro in isolated muscles. Increased glucose transport activity is therefore unlikely to be due to increased GLUT4 expression.

Certainly, and as previously reported (1, 18, 20, 24, 33), development of the obese and diabetic phenotypes depends on the mouse genetic background. For instance, CD1 mice fed the same HFD as the C57BL/6J mice did not develop diabetes (not shown). On the other hand, the differential metabolic adaptation to the high-fat diet observed in a population of C57BL/6J mice cannot be explained by different genetic background, because these mice are all genetically identical. Our data therefore show that, provided that a susceptible genetic background is present, dietary and so far-undefined environmental factors can combine to induce obesity and diabetes of variable severity. Our study shows that those factors could be involved in the differential regulation of glucose utilization driving the fate of glucose toward muscle utilization rather than toward adipose tissue storage. This model of high-fat feeding with well-characterized metabolic alterations occurring in a stepwise, identifiable manner will provide an important tool to study the changes in gene expression underlying these metabolic adaptations. The application of microarray technology to evaluate the corresponding changes in expressed transcript profiles will be an important goal of future research.

We thank Drs. L. Tappy, P. Vollenweider, and M. Uldry for helpful discussion, and we greatly appreciate W. Dolci for technical expertise.

This work was supported by grants from the Swiss National Science Foundation (3100–055881.98 to R. Burcelin and 31–46958.96 to B. Thorens) and from the Juvenile Diabetes Foundation International (1–1999–714 to R. Burcelin).

REFERENCES


25. Vaag A, Alford F, and Beck-Nielsen H. Intracellular glucose and fat metabolism in identical twins discordant for non-insulin-


