Involvement of Sex, Strain and Age Factors in High Fat Diet-Induced Obesity in C57BL/6J and BALB/cA Mice

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Abstract: Although a number of obesity animal models have been reported, each model possesses different characteristics of obesity, suggesting care should be taken in choosing an animal model suitable for the experimental purpose. In this report, we fed 4-(young) and 52-week-old (middle-aged) C57BL/6J (B6) and young BALB/cA (BALB/c) mice with a high fat diet (HFD) for 9 weeks, and investigated the clinical and histological characteristics of obesity. In BALB/c mice, males gained more body weight and body fat weight and had higher energy intake than females by HFD feeding. Comparing the effect of HFD feeding between the strains of mice, BALB/c male mice accumulated more hepatic lipid than B6 male mice. In addition, middle-aged B6 mice increased the ratio of fat to body weight and hepatic lipid accumulation more than young mice. In conclusion, the characteristics of obesity induced by HFD feeding were influenced by the sex, strain and age of mice. Sex steroid hormones, hepatic lipid metabolism and systemic metabolism might be involved in these factors. The basic data in this study will be useful for the development of animal models of high fat diet-induced obesity.

Key words: age, high fat diet, obesity model, sex, strain

Introduction

Metabolic syndrome is characterized by a group of metabolic risk factors in one person, including abnormal obesity, hypertension, hyperlipidemia and hyperglycemia, and easily leads to cardiovascular diseases. The syndrome affects a considerable number of people in many developed countries. Obesity is the most crucial factor in abnormal metabolism. Therefore, a great number of reports dealing with obesity have been published, and various animal models have been used in research.

For example, \textit{ob/ob} mice [4, 7] and \textit{db/db} mice [4, 6] have been used as animal models of obesity caused by a single gene mutation, as they are helpful to determine the role of each mutation gene in the pathogenesis of obesity. Another type of animal model is dietary-induced, for example, by feeding with a high fat diet (HFD). Obesity in humans generally involves the interaction of multiple genes and environmental factors.

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Dietary fat is considered one of the most important environmental factors in the pathophysiology of obesity; therefore, HFD-induced obesity models may well reflect human obesity conditions.

There are obesity-prone (C57BL/6J, DBA/2J), obesity-resistant (SWR/J, A/J), and intermediate (BALB/cByJ, C3H/HeJ and C57L/J) mouse strains [1, 18]. C57BL/6J (B6) mice are commonly used for obesity research, because they are lean when they are fed a low fat diet, while they show obesity characteristics such as increasing body fat mass, hyperglycemia, and hyperinsulinemia when they are fed HFD [12, 15]. BALB/cByJ mice eat less fat than B6, when they can freely access fat, protein or carbohydrate [14]. BALB/cJ, which is another BALB/c substrain, has been used to investigate the genetic factors of diabetes and insulin resistance such as the ob/ob gene [11]. Therefore, it is predicted that HFD-induced obesity of the BALB/c strain may be milder than that of B6 mice, but there were few reports about HFD-feeding BALB/c strain mice.

Obesity characteristics are also influenced by sex. In humans, males show a central pattern of fat accumulation, whereas females have a subcutaneous pattern. Energy homeostasis is also different between males and females. The sensitivity of the brain to insulin and leptin shows a sex difference [19].

In aged individuals, energy expenditure may decrease, and the ability to regulate energy intake may decline; therefore, older animals more easily develop obesity when they are fed HFD than younger ones. There are many reports dealing with such age effects related to obesity [3, 8, 13, 16, 17, 20].

Considering the above issues, it seems that the pathophysiology of obesity is closely associated with the sex, strain and age of animals. In this study, we fed B6 and BALB/c mice with HFD for 9 weeks, and investigated the involvement of the sex, strain and age of mice in the clinical and histological characteristics of obesity. The data obtained in this study will be useful for the development of animal models of obesity.

<table>
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<tr>
<th>Table 1. Composition of diets</th>
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<td>Nitrogen-free extract (%)</td>
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<td>Energy (kcal/100g)</td>
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<td>Fat-derived energy (%)</td>
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<td>Protein-derived energy (%)</td>
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Materials and Methods

Animals

We used 4-week-old (young) and 52-week-old (middle-aged) male and female B6 mice. To examine strain differences, we used young male and female BALB/c mice of the same age. All mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Animals were individually housed in plastic mouse cages. They were maintained under 14:10-h light:dark conditions. For acclimatization, young mice were fed standard rodent chow (CE-2, CLEA Japan Inc., Tokyo, Japan) and water ad libitum for 1 week, and middle-aged mice were acclimatized for 2 weeks. The mice were then separated into two groups. One was fed HFD (High Fat Diet 32, CLEA Japan Inc., Tokyo, Japan), and the other (control) was fed a standard diet (CE-2) for 9 weeks. HFD is served as a pellet and can be handled more easily than conventional powdered high fat diets.

The compositions of the two diets are shown in Table 1. Animals were weighed every week and glucose tolerance tests were performed at 5 and 9 weeks after the start of feeding (WAF). At 9 WAF, after the glucose tolerance test, mice were exsanguinated from the caudal vena cava under ether anesthesia, and were autopsied. Major organs, including the liver, kidney, spleen, heart, pancreas, lung, stomach, intestines, brain, skin, muscle, testis, ovary and abdominal and subcutaneous fat, were collected.

Calculation of energy intake

Diet intake was obtained by subtracting the weight of the remaining diet from the initially supplied diet. Energy intake was calculated on the basis of 3.404 kcal/g in the CE-2 diet and 5.068 kcal/g in HFD.

Glucose tolerance test

At 5 and 9 WAF, glucose tolerance tests were performed. Mice were fasted for 20 h, and then orally administered 20% glucose solution (0.01 ml per gram
of body weight). Blood was collected from the tail veins using heparinized capillary tubes (Hematlon, Yamauchi Medical, Tokyo, Japan) before glucose administration (0 min) and at 30, 60, 90 and 120 min after administration.

The obtained blood was centrifuged at 10,000 rpm for 10 min. Plasma samples were stored at –80°C until use. Plasma glucose concentrations were determined with Glucose CII-test Wako (Wako, Osaka, Japan). For assessing glucose tolerance, the area under the curve (AUC) on the tolerance chart was calculated by the following formula:

\[ AUC = \frac{(A + B \times 2 + C \times 2 + D \times 2 + E)}{2} - A \times 4 \]

- A: 0 min glucose concentration
- B: 30 min
- C: 60 min
- D: 90 min
- E: 120 min

Lower AUC values reflect more efficient glucose clearance.

Blood biochemistry

Serum samples were prepared from the total blood collected via the caudal vena cava by centrifugation at 2,000–3,000 rpm for 10 min. Using commercial kits, serum cholesterol (Cholesterol E-test Wako, Wako, Osaka, Japan), serum triglyceride (Triglyceride E-test Wako, Wako, Osaka, Japan) and serum free fatty acid (NEFA C-test Wako, Wako, Osaka, Japan) were measured according to the manufacturer’s instructions.

Histopathological examination

The major organs and fat (subcutaneous, perirenal, mesenteric and perireproductive) were weighed and fixed in 10% neutral-buffered formalin. Tissue samples were embedded in paraffin and 4-µm paraffin sections were stained with hematoxylin and eosin (HE). To estimate the extent of hepatic lipid accumulation, the distribution of lipid vacuoles in liver sections was scored. First, ten hepatic lobules in which both central vein and portal tract were clearly observed were chosen from liver sections of each animal. Then, the distribution of lipid accumulation was scored according to three zones (zone1, perilobular; zone2, intermediate; zone3, pericentral) in a hepatic lobule. That is, the number of zones (0–3) with lipid accumulation was used as the score of lipid distribution, and the scores of ten lobules were averaged for each animal. Finally, the average of each group was calculated using constituent animal scores.

Statistical analysis

Results are presented as the mean ± standard deviation (SD). For statistical analyses, Student’s t-test was used for HFD groups vs. control groups.

Results

Energy intake per day

The food intake per day of HFD-fed mice was significantly less than that of control mice (Table 2). The energy intake per day of HFD-fed mice was significantly larger than that of control mice in all male groups and middle-aged B6 female groups, but not in young B6 and BALB/c female groups (Table 2). Energy intake per day was not significantly different between the control group and HFD group in young BALB/c female mice, whereas it was 20% higher in the young BALB/c male HFD group than in the control group. In the young B6 male HFD group, the intake was 16% higher than that of the control, whereas it was 31% higher in the middle-aged B6 male HFD group.

Body weight

Body weight gain was not different between HFD and control groups of young BALB/c females (Fig. 1D). In the middle-aged B6 female group, the body weight of HFD-fed mice was always higher than that of control mice, but the difference was not significant (Fig. 1F). In the other HFD groups, body weights of HFD mice began to increase significantly at 1–2 WAF compared with those of control mice, and then continued to be higher (Fig. 1A–C, E).

Fat accumulation and the ratio of fat to body weight

The total fat weight (subcutaneous and visceral fat) of all HFD groups was significantly higher than the control, except for young BALB/c females (Fig. 2A). The increase of fat weight caused by HFD intake was lower in young BALB/c female (2.9-fold) than the other young groups (young BALB/c male, 6.9; young B6 male, 7.1 and young B6 female, 10.5).

The ratio of fat to body weight significantly increased in HFD-fed mice compared to control mice (Fig. 2B). The HFD-induced increase of the fat-body weight ratio was lower in young BALB/c females (2.22-fold) than in the other young groups (young BALB/c male, 5.07; young B6 male, 4.30 and young B6 female, 6.92).
Liver weight

The liver weight of HFD mice was significantly higher than the control in BALB/c males and middle-aged B6 males (Fig. 3). Compared to the liver weight of each control group, the weight of HFD mice increased only 5% in young BALB/c females, whereas the increase was 44% in young BALB/c males, 12% in young B6 males and 87% in middle-aged B6 males.

Glucose tolerance test

Plasma glucose concentration at the glucose tolerance test of 9 WAF is shown in Fig. 4A–F. Plasma glucose concentrations of HFD groups after glucose solution administration tended to be higher than that of control groups. AUC calculated at 9 WAF is shown in Fig. 4G. AUC was significantly higher for the middle-aged B6 female HFD group than for the control group (Fig. 4G). AUCs of young BALB/c female HFD and
the control group were almost the same. In the other groups, AUC tended to be higher in HFD-fed mice than in the control, but not significantly. AUC at 5 WAF was similar to AUC at 9 WAF (data not shown).

**Blood biochemistry**

The serum free fatty acid level was not significantly different between HFD and control mice (data not shown). The serum triglyceride level of the HFD group was significantly higher than that of the control group only in young B6 females (Fig. 5A). The serum total cholesterol level of HFD mice was significantly higher than that of the control in all groups (Fig. 5B).

**Histopathological examination**

Increased lipid accumulation was observed in the livers of all HFD groups, except for young BALB/c female. To estimate the extent of hepatic lipid accumulation, the distribution of lipid vacuoles in hepatic lobules was scored (Table 3). Because hepatic lipid accumulations were not observed in all control mice and young BALB/c female HFD-feeding mice, the score was not calculated for these groups. According to the score, the severity of hepatic lipid accumulation caused by HFD followed this order: middle-aged B6 male and female > young BALB/c male > young B6 male and female > young BALB/c female (Table 3, Fig. 6). BALB/c female HFD mice showed no hepatic lipid accumulation. The lipid vacuole distribution patterns were centrilobular in young BALB/c males and middle-aged B6 males (Fig. 6E, G), and perilobular in middle-aged B6 females (Fig. 6H).

Increased lipid accumulation was also observed in renal proximal convoluted tubules of B6 male HFD mice and subcutaneous tissue of all HFD mice (data not shown). In the other tissues (spleen, heart, pancreas, lung, stomach, intestines, brain, muscle, testis and ovary), there were no remarkable histopathological changes.

**Discussion**

In the present study, B6 and BALB/c mice were fed HFD for 9 weeks. HFD feeding increased body fat accumulation and induced obesity conditions in the animals. It was also shown that HFD feeding induced diverse characteristics of obesity according to the sex, strain and age differences of the mice. Specially, we showed that 1) young BALB/c female mice were resistant to HFD-induced obesity compared to males; 2) young B6 males and young BALB/c males became
equally obese, however, hepatic lipid accumulation was different between these two strains; and 3) middle-aged B6 mice were more prone to HFD-induced obesity than young B6 mice.

This study was performed using the obesity-prone B6 mice strain, to compare the effects of sex, strain and age on HFD-induced obesity. To examine the strain effect, BALB/c mice were picked as counterparts of B6 mice. This is because BALB/c is an intermediate phenotype between the obesity-prone and resistant strains, and because there are few reports about high fat diet-induced obesity in BALB/c mice. To examine the age effect, young and middle-aged B6 mice were compared in this study. The age effect in BALB/c mice was not examined in this study. For the comparison of sex, strain and age, the extent of the difference between HFD and control diet in each sex, strain and age group was compared.

**Fig. 4.** Glucose tolerance test at 9 WAF (n=3 to 5). Plasma glucose of (A) Young B6 male, (B) young B6 female, (C) young BALB/c male, (D) young BALB/c female, (E) middle-aged B6 male, and (F) middle-aged B6 female mice. (G) AUC. Values are the mean ± SD. Significant differences are indicated as * (HFD vs. control) at P<0.05.
Sex factors

In young BALB/c mice, males indicated moderate obesity due to HFD feeding, whereas females showed slight obesity at 9 WAF. In male mice, HFD increased body weight, body fat weight and hepatic lipid compared to female mice. This result indicates that BALB/c female mice are comparatively resistant to HFD-induced obesity compared to males. The increase in energy intake of young female BALB/c mice due to HFD feeding (5%) was lower than that of young male BALB/c mice (20%), suggesting that lower energy intake in HFD-fed BALB/c females than males may lead to the observed resistance to obesity. On the other hand, in young B6 mice, the HFD-induced increase of energy intake was much the same in both sexes and the difference was not significant (male, 16% and female, 12%). In addition, the characteristics of obesity in HFD females were equivalent to those in males. Considering these results, it is suggested that BALB/c mice would be more suitable for experiments investigating the differences in HFD-induced obesity between males and females.

In this study, although the mechanism underlying the sex differences remains unclear, the different energy intake would have partly caused the sex differences observed in BALB/c mice, since food intake is influenced by sex [5, 10, 19].

Strain factors

Qiu et al. [11] showed that abnormalities caused by leptin deficiency in ob/ob mice can be improved by the genetic background of BALB/cJ. This indicates that BALB/cJ mice have modifier genes which can rescue a defective leptin pathway. In the experiments of Alexander et al. [1] and Smith et al. [14], it was shown that BALB/cByJ mice ate less fat than B6 mice even when they were able to freely choose nutrients (protein, fat and carbohydrate), and body fat weight was lower in BALB/cByJ than in B6 mice.

In this study, the HFD-induced increase of the fat-body weight ratio was almost equal in young B6 and BALB/c males; however, HFD-induced increase of hepatic lipid vacuoles and liver weights were markedly increased in BALB/c males.

The different increase of hepatic lipid accumulation between BALB/c and B6 males may be related to differences in hepatic lipid metabolism. Lin et al. [9] reported that hepatic lipid accumulation is different among strains (BALB/c, C57BL and SWR) and is influenced by the rate of hepatic fatty acid synthesis, triglyceride secretion and triglyceride oxidation in each strain. Therefore, the hepatic lipid metabolism is potentially different between B6 and BALB/c male mice. HFD may amplify this difference. Lipid accumulation in the liver depends on the balance of synthesis, transport and removal of fatty acids and triglycerides [2]. Our data showed that serum triglyceride and free fatty acid were not significantly different between control and HFD mice in both B6 and BALB/c mice and HFD-induced increase of cholesterol levels was almost equal in these two strains; therefore, it remains unclear which metabolic pathway is involved in the differences of hepatic fat accumulation observed in the two strains in this study.

![Fig. 5. Blood biochemistry at 9 WAF (n=5). (A) Serum triglyceride and (B) cholesterol levels. Values are the mean ± SD. Significant differences are indicated as * and ** (HFD vs. control) at P<0.05 and P<0.01, respectively.](image-url)
Fig. 6. Histopathological findings of the liver at 9 WAF. A: middle-aged B6 male control mouse; B: middle-aged B6 female control mouse; C: young B6 male HFD mouse; D: young B6 female HFD mouse; E: young BALB/c male HFD mouse; F: young BALB/c female HFD mouse; G: middle-aged B6 male HFD mouse; and H: middle-aged B6 female HFD mouse. Scale Bar = 100 µm. Hematoxylin & eosin.
Age factors

In young and middle-aged B6 mice, HFD increased body weight and fat-body weight ratio and decreased glucose tolerance, compared to control diet. Contrary to our expectation, these changes were larger in young B6 mice. Regarding the reason for this, we think that the middle-aged control mice were already obese to some extent. Indeed, in comparing the young B6 control mice with middle-aged B6 control mice, the body weight and fat-body weight ratio of the middle-aged mice were higher than those of the young mice. Therefore, the middle-aged B6 mice were basically obese compared to the young B6 mice. Some reports have shown that body fat accumulation increases in aged rodents and humans [3, 17, 20]. This increase may be due to the change of physical activity [16, 17] and systemic metabolism, such as reduction of systemic energy expenditure, insulin sensitivity and leptin sensitivity [8, 13]. Moreover, these changes in middle-aged mice would amplify the effect of HFD in induction of obesity. Therefore, in this experiment, we think that age-related changes may have induced severe obesity, including body weight, fat-body weight ratio and hepatic lipid increase, in middle-aged B6 HFD-fed mice, comparing with young B6 HFD-fed mice.

In the result of glucose tolerance test, the AUC of middle-aged mice was lower than that of young mice, showing conflict with the results discussed above. Although the reason for this is still not clear at this time, detailed examination of this point is planned in further research.

In conclusion, sex, strain and age factors are involved in the modification of hepatic lipid metabolism and obesity conditions induced by HFD feeding.

Table 3. Score of hepatic lipid accumulation

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<tr>
<th>Mouse strain &amp; age</th>
<th>Score of hepatic lipid accumulation±</th>
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<tr>
<td>Young B6 male HFD</td>
<td>1.07 ± 0.59</td>
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<tr>
<td>Young B6 female HFD</td>
<td>1.1 ± 0.36</td>
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<tr>
<td>Young BALB/c male HFD</td>
<td>2.12 ± 0.17</td>
</tr>
<tr>
<td>Young BALB/c female HFD</td>
<td>ND</td>
</tr>
<tr>
<td>Middle-aged B6 male HFD</td>
<td>2.62 ± 0.31</td>
</tr>
<tr>
<td>Middle-aged B6 female HFD</td>
<td>2.4 ± 0.36</td>
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a: values are the mean ± SD (n=5–6). b: ND; not done.

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References

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