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Sex difference of hyperinsulinemia in the C57BL/6J-Daruma (obese) mouse

Yoshihiro SUZUKI[1)], Keiko NAKAHARA[2)]*, Takuya ENSHO[2)] and Noboru MURAKAMI[2)]

[1)]Division of Animal Science, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan
[2)]Department of Veterinary Physiology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan

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ABSTRACT. The C57BL/6J-Daruma mouse is an animal model of obesity derived from the original genetically obese ICR-Daruma mouse by transfer of the phenotype into the C57BL/6J background by backcrossing into the C57BL/6J strain. Although, like the original ICR-Daruma mouse model, both male and female C57BL/6J-Daruma mice develop obesity, the latter strain shows sex differences in several phenotypes. A sex difference in plasma insulin levels was especially notable in C57BL/6J-Daruma mice; only males showed hyperinsulinemia. Orchiectomy suppressed this hyperinsulinemia completely, whereas testosterone supplementation restored it. Glucose administration increased the plasma glucose level in both male and female Daruma mice to a greater extent than in wild-type control mice. Orchiectomy, but not ovariectomy, decreased the plasma glucose level to that seen in wild-type controls. On the other hand, this effect of orchiectomy was abrogated by testosterone supplementation. The expression of mRNAs for several genes related to insulin resistance was significantly changed in white adipose tissue and liver of C57BL/6J-Daruma mice, especially males, as early as 4 weeks of age. The present results suggest that testosterone may be involved in the hyperinsulinemia shown by male C57BL/6J-Daruma mice and that this strain may be an appropriate animal model for examining the relationship between obesity and sex hormones.

KEY WORDS: hyperinsulinemia, hyperleptinemia, insulin resistance, novel monogenic obese mice, sex difference

Obesity is a disorder of energy balance resulting from excessive energy intake and/or reduced energy expenditure. This induces hypertrophy of white adipose tissue (WAT). Many studies have indicated that obesity is a risk factor for type 2 diabetes, stroke and myocardial infarction [1, 10, 18]. Insulin, a hypoglycemic hormone, and leptin, an anorexigenic hormone that promotes energy expenditure, have been considered to play a crucial role in obesity [4, 20, 29]. It is well known that obese individuals often develop insulin resistance and/or leptin resistance. Tumor necrosis factor-α (TNF-α), free fatty acid (FFA) and resistin secreted from WAT are known to play a role in insulin resistance [27], and their blood levels are increased with WAT hypertrophy in obese individuals. Obese patients with insulin resistance develop hyperinsulinemia in order to maintain normal blood glucose levels. This leads to exhaustion of pancreatic beta-cells, and type 2 diabetes finally develops as a result of insulin hyposecretion. Obese individuals show a reduction in the capacity of leptin to affect appetite and energy expression, despite the fact that blood leptin levels are increased with WAT hypertrophy [12, 22], a condition known as "leptin resistance". In fact, administration of exogenous leptin to obese individuals does not lead to decrease in food intake [12].

Studies of obesity have generally employed genetically obese mice and/or diet-induced models of obesity. Genetically obese mice, such as the lethal yellow agouti (*Ay/a*) [3, 19], fat (*fat/fat*) [3, 10, 11, 33], tubby (*tub/tub*) [9, 32], obese (*ob/ob*) and diabetic (*db/db*) strains [6, 17], have been widely studied as models of monogenic obesity. We have previously reported the establishment of a novel strain of genetically obese mice, ICR-Daruma [26]. These mice developed characteristic visceral fat accumulation at 4 weeks of age, and the WAT and liver exhibited increases in cell size and lipid droplets, respectively. Moreover, the mice exhibited early onset of impaired leptin signaling, manifested as hyperleptinemia, and hyperinsulinemia. The ICR-Daruma mouse has two base mutations in the leptin receptor gene sequence that are good candidates for the variation(s) responsible for the obese phenotype. No obesity was observed in pups resulting from a cross between heterozygous ICR-Daruma mice and heterozygous *ob/+*, tubby or fat mice. On the other hand, 13% of 82 pups produced by crossing heterozygous ICR-Daruma mice with heterozygous *db/+* mice developed obesity, suggesting that various mechanisms of interaction may exist. Accordingly, Daruma mice exhibit unique characteristics and may be a good model for studies of human metabolic syndrome.

*Correspondence to: Nakahara, K., Department of Veterinary Physiology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2155, Japan. e-mail: a0d209u@cc.miyazaki-u.ac.jp

In addition, we have succeeded in transferring the phenotype of the ICR-Daruma mouse to a C57BL/6J background. C57BL/6J-Daruma mice were back-crossed 13 generations into the C57BL/6J strain without loss of ICR-Daruma mouse characteristics. However, unlike the ICR-Daruma mice, C57BL/6J-Daruma mice exhibited some unique characteristics, such as a clear sex difference in blood insulin levels. Here, we report some of the characteristics of the C57BL/6J-Daruma mouse strain.

MATERIALS AND METHODS

Animals

C57BL/6J-Daruma mice were produced by transfer of the ICR-Daruma phenotype [26] to a C57BL/6J background through 13 generations. C57BL/6J-Daruma mice used in this study were originally purchased from Japanese Charles River (Yokohama, Japan). They were JAX Mice (stock number 000664) and not in the substrain diverged genetically. Mice with plasma leptin concentrations exceeding 7 *ng*/m*l* at 3 weeks of age and those with mutations at two base positions in exons 8 and 15 of the leptin receptor gene were designated as C57BL/6J-Daruma mice, and the other mice were assigned to a wild-type control group. All mice were housed under constant temperature (23 ± 1°C) and a 14L: 10D lighting regime (lights on at 05:00 hr) with free access to food and water. To obtain growth curves and to monitor food intake, body weight and food intake of male and female C57BL/6J-Daruma mice and control mice were measured once a week from weaning until 53 weeks of age and once a week from weaning until 16 weeks of age, respectively.

All experiments were conducted in accordance with the Japanese Physiological Society's guidelines for animal care, and the experiments were authorized by Miyazaki University Animal Experiment Committee (authorization number: 2006-051-1~6).

Blood sampling and biochemical analysis

To compare the biochemical properties of blood in intact male and female C57BL/6J-Daruma and wild-type control mice, blood samples were collected by tail tip incision once a week or at biweekly intervals. A proportion of the mice were fasted for 16 hr from 18:00 to 10:00 hr before the sampling day, in order to examine the fasting blood glucose level. Glucose, triglyceride and total cholesterol in plasma were measured using a commercial kit: DRI-CHEM3500V (Fuji Medical Systems Co., Ltd., Tokyo, Japan). Plasma insulin and leptin concentrations were measured using a mouse insulin ELISA kit (Morinaga Co., Ltd., Yokohama, Japan) and a mouse leptin ELISA kit (Morinaga Co., Ltd.) following each of the manufacturers' protocols.

Gonadectomy and steroid supplementation

A proportion of the male and female C57BL/6J-Daruma mice underwent bilateral removal of the testes and ovaries at 7 weeks of age under anesthesia, followed by subcutaneous implantation of a silicone tube (8 mm long, 2 mm ID and 3.5 mm OD) filled with testosterone (for males) or estradiol 17β (for females). These steroid implants maintained blood testosterone and estrogen at normal levels for more than 3 months. Blood samples were collected from these gonadectomized C57BL/6J-Daruma mice with steroid implants once a week from 7 to 16 weeks of age for measurement of glucose, insulin and leptin levels.

Glucose and insulin tolerance testing

Glucose and insulin tolerance tests were performed in intact or gonadectomized C57BL/6J-Daruma mice at 9 weeks of age. All mice were fasted for 16 hr from 18:00 to 10:00 hr. Glucose (1.5 mg/g body weight) or insulin (0.75 mU/g body weight) was administered at 10:00 hr intraperitoneally. After injection, blood samples were collected at 0, 15, 30, 60, 120 and 180 min in glucose tolerance test, and at 0, 20, 40, 60, 80, 100 and 120 min in insulin tolerance test. Plasma samples were immediately used for analysis of glucose and insulin levels.

Expression of mRNAs for peptides in the liver and WAT related to insulin resistance

After removal of the liver and WAT of male and female C57BL-Daruma and wild-type control mice at 4 weeks of age, total RNA was immediately extracted using Trizol (Life Technologies Co., Carlsbad, CA, U.S.A.) and purified using an RNeasy plus Micro kit (Qiagen, Valencia, CA, U.S.A.). Total RNA (1 μg in a final volume of 111 μ*l*) was reverse-transcribed into first-strand complementary DNA (cDNA) using a RT^2 First Strand Kit (Qiagen). PCR was carried out using a Mouse Insulin Resistance RT^2 Profiler PCR Array (Qiagen) for 84 genes.

Histological examination

Histological analysis was performed on male and female C57BL/6J Daruma and control mice at 9 weeks of age. Liver and WAT were removed after decapitation, then fixed in 4% paraformaldehyde-0.1 M phosphate-buffered saline (PBS) for 7 days and transferred to 0.1 M PBS. After being embedded in paraffin, they were cut into 10 μm thick sections and stained with hematoxylin and eosin.

Statistical analysis

The data (mean ± SE) were analyzed statistically by ANOVA with the *post hoc* Fisher test.

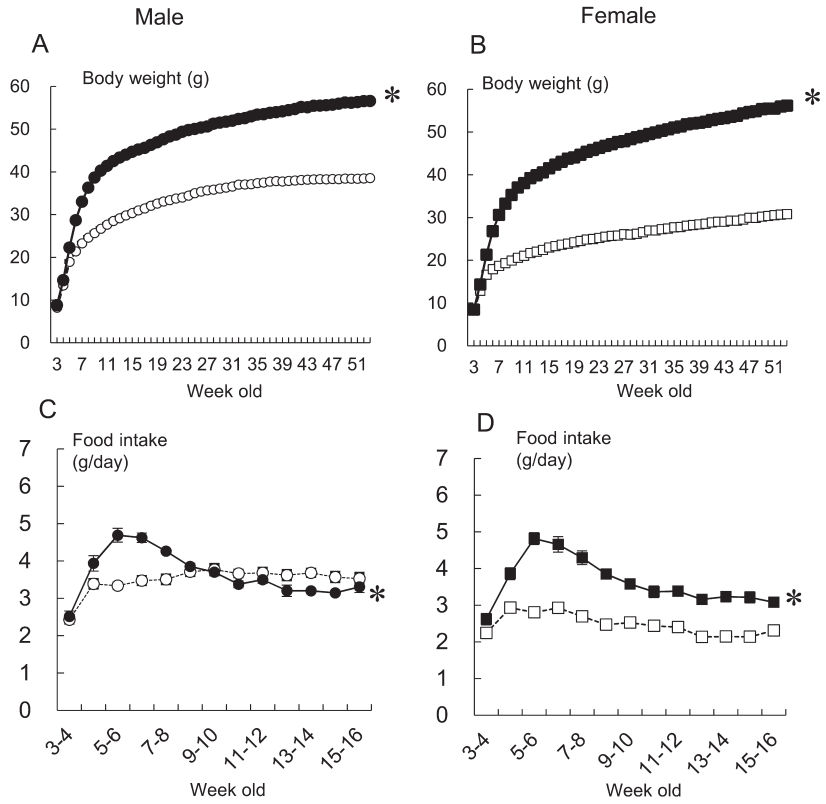

Fig. 1. Comparison of growth curve and food intake between female and male C57BL/6J-Daruma mice and control wild-type mice. A, B: growth curves of C57BL/6J-Daruma (●: male n=18, ■: female n=18) and control wild-type (○: male n=15, □: female n=29) mice. C, D: food intake of C57BL/6J-Daruma (●: male n=12 ■: female n=10) and control wild-type (○: male n=12, □: female n=10) mice. Each symbol and vertical lines represent the mean ± SE. **P*<0.05; C57BL/6J-Daruma mice vs control wild-type mice.

RESULTS

The body weight of male and female C57BL/6J-Daruma mice exceeded that of wild-type control mice between 5 and 6 weeks of age, and reached about 1.5- and 2-fold at 11 weeks, respectively (Fig. 1A and 1B). The difference in growth rate between Daruma mice and wild-type control mice was greater in females than in males. Although hyperphagia was observed in both male and female Daruma mice from 4 weeks of age, food intake returned to normal at 8 weeks of age in males (Fig. 1C and 1D). On the other hand, females continued to exhibit hyperphagia.

Some biochemical characteristics of male and female C57BL/6J-Daruma mice are shown in Fig. 2. Although the plasma concentrations of glucose under *ad libitum* feeding in both male and female Daruma mice were not significantly different from those of wild-type control mice (Fig. 2A and 2G), plasma glucose levels under fasting conditions were significantly higher (Fig. 2B and 2H). Blood triacylglycerol levels in both male and female Daruma mice did not differ significantly from those in wild-type control mice (Fig. 2C and 2I). On the other hand, the levels of total cholesterol were higher in both male and female Daruma mice than in wild-type control mice (Fig. 2D and 2J). With regard to blood insulin levels, a clear difference was observed between male and female Daruma mice (Fig. 2E and 2K); there was a gradual increase with growth in males, but not in females. In females, a slightly but significantly high level of insulin continued from 6 until 16 weeks of age. On the other hand, leptin levels rose rapidly until about 7 weeks of age and then rose gradually thereafter in both males and females (Fig. 2F and 2L).

To investigate the reason for the sex difference in blood insulin levels, we first examined the effect of gonadectomy and/or steroid hormone supplementation on blood insulin levels in both male and female C57BL/6J-Daruma mice. In males, orchiectomy resulted in total atrophy of the seminal vesicles and congealing glands, and this was abrogated by continuous administration of testosterone from the silastic implant (Fig. 3A). In addition, orchiectomy significantly damped the growth curve, whereas testosterone supplementation restored the growth curve to that observed in Daruma mice that had undergone a sham operation (Fig. 3B). Blood glucose levels were decreased slightly in orchidectomized Daruma mice relative to those that had undergone a sham operation or been orchidectomized followed by testosterone supplementation. Interestingly, orchiectomy completely abrogated any increase of the blood insulin level in Daruma mice. On the other hand, orchidectomized Daruma mice supplied with testosterone showed an increase of insulin similar to that in Daruma mice that had undergone a sham operation (Fig. 3D).

In female C57BL/6J-Daruma mice, ovariectomy alone (without estradiol 17β supplementation) did not significantly alter the growth curve, or plasma glucose and insulin levels, in comparison with those that had undergone a sham operation (Fig. 4). On the

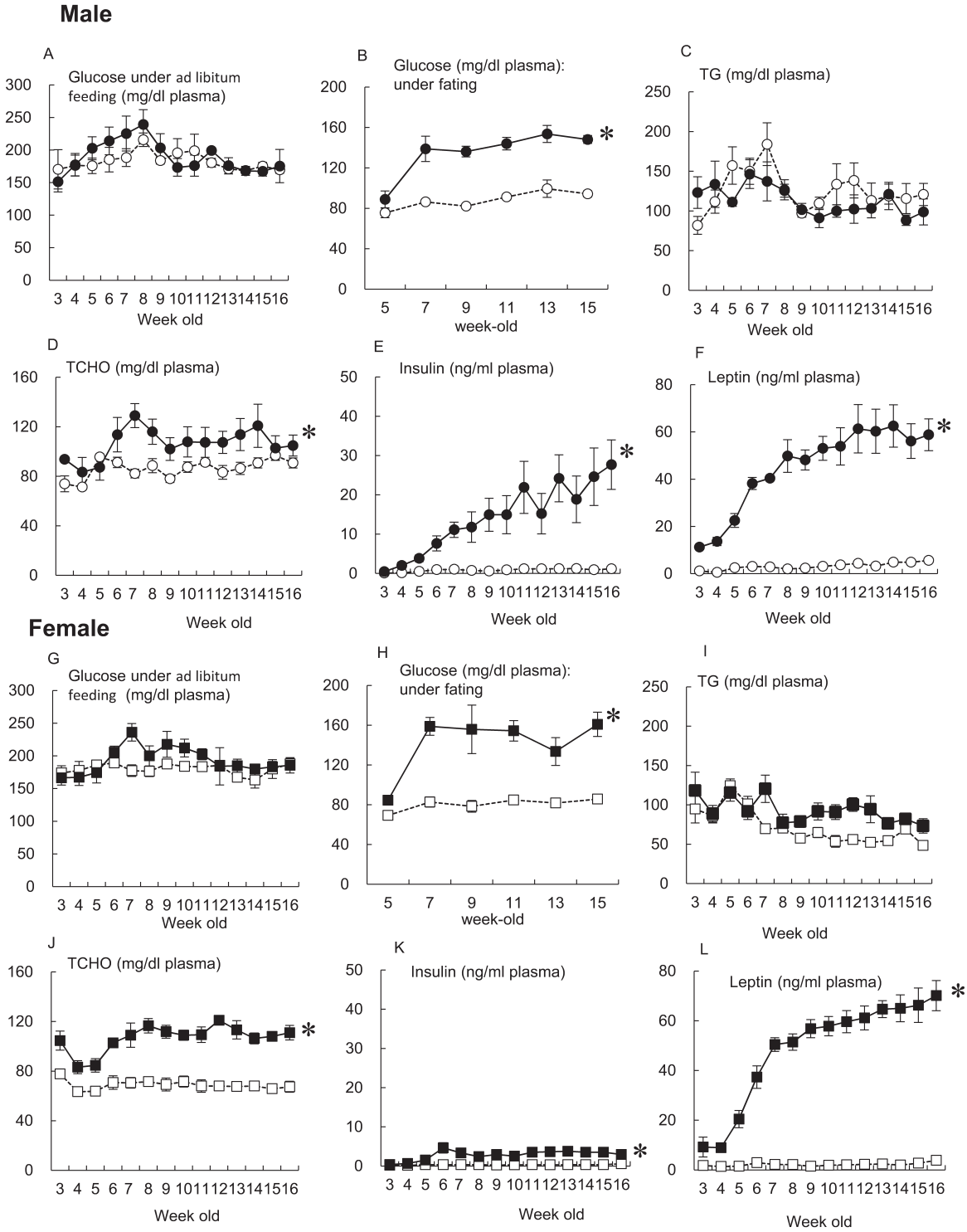

Fig. 2. Blood biochemistry of C57BL/6J-Daruma mice. A, G: Glucose under *ad libitum* feeding, B, H: Glucose under fasting, C, I: Triglyceride, D, J: Total cholesterol, E, K: Insulin, F, L: Leptin in male and female C57BL/6J-Daruma (male n=12, female n=12) and control wild-type (male n=10, female n=10) mice. White and black symbols indicate control wild-type and C57BL/6J-Daruma mice, respectively. Each of the symbols and vertical lines represent the mean ± SE *P<0.05; C57BL/6J-Daruma mice vs control wild-type mice.

other hand, continuous administration of estradiol 17β via a silastic implant following ovariectomy dampened the growth curve and slightly decreased the plasma glucose and insulin levels in comparison with ovariectomized Daruma mice or those that had undergone a sham operation (Fig. 4A–C). In spite of ovariectomy and/or estrogen administration, however, plasma insulin levels in female Daruma mice did not exceed 20 *n*g/m*l*, as had been observed in male Daruma mice (Fig. 4C).

Next, we compared the responses of male and female C57BL/6J-Daruma mice to glucose and insulin tolerance tests (Figs. 5

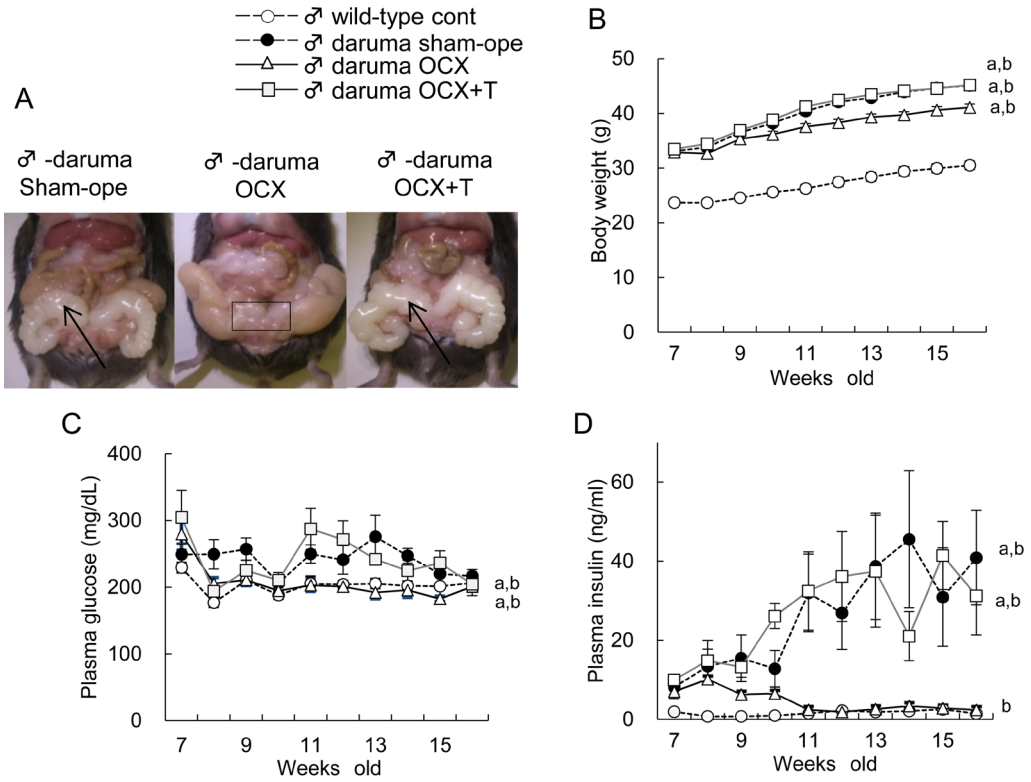

Fig. 3. Effect of orchiectomy (OCX) and testosterone (T) supplementation on the growth, blood glucose and insulin levels in male C57BL/6J-Daruma mice. A: Photograph of seminal vesicle and congealing gland in C57BL/6J-Daruma mice subjected to a sham operation (sham-op), or orchiectomy with or without testosterone supplementation. Arrow and square box indicate the seminal vesicle and congealing gland. B: Body weight, C: Plasma glucose, D: Plasma insulin in the four groups. Each of the symbols and vertical lines represent the mean ± SE (n=8). **P*<0.05; a: Daruma sham-op vs wild-type control, Daruma OCX vs wild-type control, Daruma OCX+T vs wild-type control; b: Daruma sham-op vs Daruma OCX, Daruma OCX+T vs Daruma OCX.

and 6). After glucose administration, the blood glucose concentration increased significantly at 15 and 30 min, and then returned to the pretreatment level by 180 min in both wild-type and Daruma mice (Fig. 5A and 5C). However, male Daruma mice that had undergone a sham operation or been orchidectomized and received a testosterone implant showed higher glucose levels at 30 and 60 min than wild-type mice or orchidectomized Daruma mice without testosterone supplementation (Fig. 5A). In Daruma mice that had undergone a sham operation or been orchidectomized followed by testosterone supplementation, insulin levels continued to increase until 120 min after glucose administration and remained high for 180 min. In orchidectomized Daruma mice without testosterone supplementation, a higher increase than the other groups was observed until 60 min (Fig. 5B). In female Daruma mice that had undergone a sham operation or been ovariectomized without estrogen supplementation, glucose levels were higher at 30 and 60 min than in wild-type mice or ovariectomized Daruma mice supplemented with estrogen (Fig. 5C). In female Daruma mice that had undergone a sham operation or been ovariectomized without estrogen supplementation, insulin levels higher than those of wild-type mice were observed from 15 until 120 min after glucose administration. In ovariectomized female Daruma mice bearing estrogen implants, insulin levels became higher than those in wild-type mice at 15 and 30 min, and then returned to the basal levels at 120 min (Fig. 5D).

Although insulin administration decreased the plasma glucose level in all wild-type and Daruma mice, the decrease was larger in wild-type than in Daruma mice irrespective of sex (Fig. 6A and 6C). There was no significant difference in the effect of insulin administration on plasma glucose and insulin levels between Daruma mice that had undergone a sham operation and those that had been gonadectomized with or without steroid supplementation (Fig. 6A–D).

PCR array analysis revealed that as early as 4 weeks of age, C57BL/6J-Daruma mice showed altered levels of mRNA expression for several genes related to insulin resistance in WAT and liver (Fig. 7A and 7B). In the liver, expression of mRNA for CD36 antigen (CD36), stearoyl-coenzyme A desaturase 1 (SCD1) and very low density lipoprotein receptor (VLDL-R) was increased, and that for leptin receptor (OB-R) was decreased, in both male and female C57BL/6J-Daruma mice, without any sex difference. In the WAT, expression of mRNA for leptin, oxidized low density lipoprotein receptor 1 (Olr1) and VLDL-R was increased in both males and females, but leptin mRNA expression was higher in males than in females. On the other hand, in the WAT, expression of mRNA for many genes was significantly decreased in males; these included apolipoprotein E (ApoE), conserved helix-loop-helix ubiquitous kinase (Chuk), insulin receptor substrate 1 (IRS-1) and 2 (IRS-2), OB-R, phosphoenolpyruvate carboxykinase 1 (Pck1), phosphodiesterase 3B (Pde3b) and PYD and CARD domain containing (Pycard) (Fig. 7A and 7B). Although there was no change

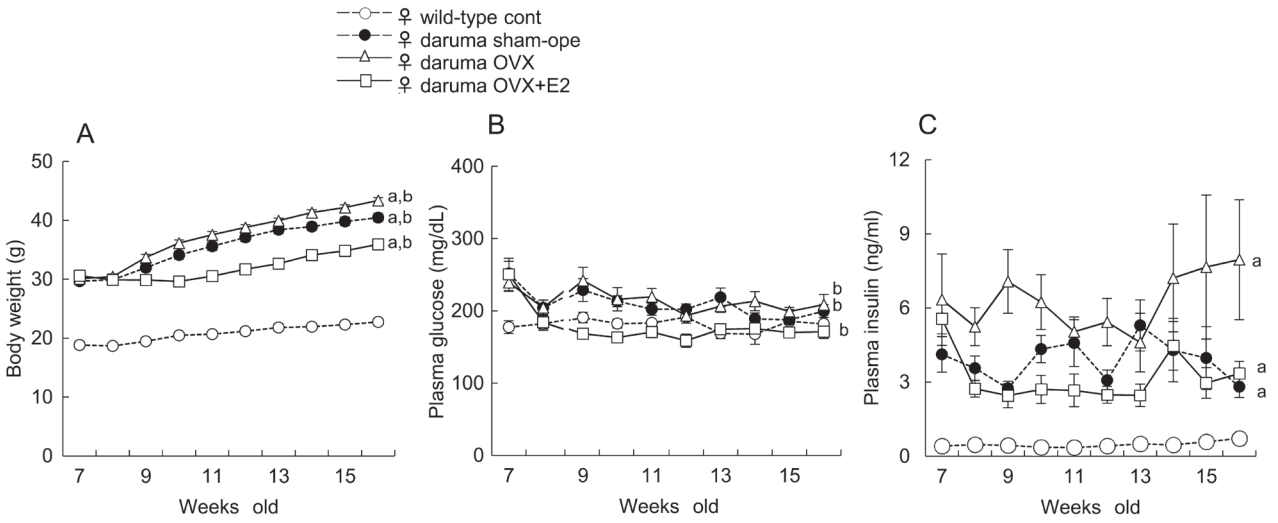

Fig. 4. Effect of ovariectomy (OVX) and estradiol 17β (E2) supplementation on the growth, blood glucose and insulin levels in female C57BL/6J-Daruma mice. A: Body weight, B: Plasma glucose, C: Plasma insulin in the four groups. Each of the symbols and vertical lines represent the mean ± SE (n=8) *P<0.05; a: Daruma sham-op vs wild-type control, Daruma OVX vs wild-type control, Daruma OVX+E2 vs wild-type control; b; Daruma sham-op vs Daruma OVX+E2, Daruma OVX vs Daruma OVX+E2.

in histological observation in liver and WAT between male and female C57BL/6J Daruma mice at 4 weeks of age (data not shown), the lipid droplet accumulation in the liver was observed at 9 weeks of age in male but not female C57BL/6J Daruma mice, and hypertrophic adipocytes in the WAT was observed in both male and female C57BL/6J Daruma mice (Fig. 7C).

DISCUSSION

In order to clarify the phenotype and characteristics of male and female C57BL-Daruma mice, we analyzed their growth curves, daily food intake, blood biochemistry parameters, glucose or insulin tolerance, and expression of mRNAs for genes related to insulin resistance in the liver and WAT.

Our previous study had demonstrated that the original ICR-Daruma mice had mutations at 2 base positions within exons 8 and 15 of the leptin receptor gene [26]. Similarly, in C57BL-Daruma mice, the leptin receptor gene was mutated in the same position. In addition, we observed that non-obese ICR mice carried the same mutations of the leptin receptor gene [26]. Therefore, these mutations of the leptin receptor gene may be not a direct cause of the obesity seen in Daruma mice. However, 26% of 486 pups produced by breeding heterozygous C57BL/6J-Daruma mice of both sexes developed obesity, suggesting that all the mutated genes are homozygous and show Mendelian inheritance. In the non-obese phenotype, in addition, there was no example of homozygosity. These results suggested that C57BL/6J-Daruma mice had monogenic obesity. Because mutation of the leptin receptor gene is linked to the obese phenotype, it is thought that the responsible gene is located on chromosome 4 with the leptin receptor gene. Further research will be needed to clarify the whole genome in order to determine the responsible gene.

In C57BL-Daruma mice of both sexes, body weight increased rapidly after 5–7 weeks of age, unlike the situation in wild-type controls. Furthermore, the body weight of C57BL-Daruma mice exceeded 40 g at 10 and 14 weeks in males and females, respectively. This excessive growth suggested that the C57BL-Daruma mice had inherited the obese phenotype from the original ICR-Daruma mice. The onset of obesity occurred at 5–7 weeks in *ob/ob* and *db/db* mice, at 6–8 weeks in *fat/fat* mice and at 10 weeks in *tub/tub* mice [6, 7, 21]. The timing of obesity onset in C57BL/6J-Daruma mice was thus close to that in other mouse strains with monogenic obesity.

Hyperphagia in male C57BL6J-Daruma mice was observed only at 4–7 weeks of age, and thereafter, normal food intake was maintained. On the other hand, hyperphagia in female C57BL6J-Daruma mice continued until end of the observation period (16 weeks). No such sex differences in the period of hyperphagia were observed in the original ICR-Daruma mice [26]. The reason for continued obesity development in male C57BL6J-Daruma mice after a return to normal food intake may have been strong suppression of energy expenditure at the time of insulin and/or leptin resistance.

As development of obesity progresses, C57BL/6J-Daruma mice of both sexes showed an increase of total cholesterol, but not triglyceride levels. In the liver, there is usually little expression of the VLDL receptor to facilitate the uptake of VLDL [28, 31]. However, in C57BL/6J-Daruma mice, expression of mRNA for the VLDL receptor in the liver and WAT was increased several-fold relative to wild-type control mice. This may have facilitated the uptake of lipid into liver tissue, so that there was no deterioration of blood biochemistry balance leading to hyperlipidemia.

The present study showed that C57BL/6J-Daruma mice of both sexes had increased plasma leptin levels in comparison with wild-type controls at 3 weeks of age, i.e. around the time of weaning. The plasma leptin levels in both sexes increased with growth

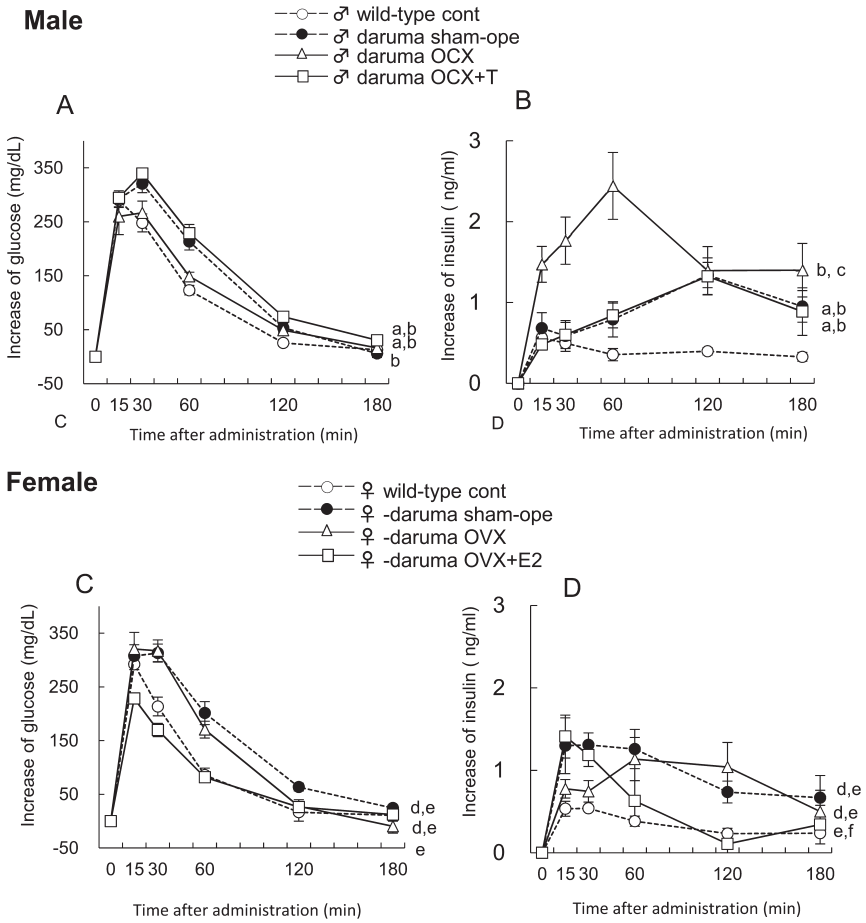

Fig. 5. Comparison of glucose tolerance test results between wild-type control mice, and female and male C57BL/6J-Daruma mice subjected to a sham operation or gonadectomy with or without steroid supplementation. Each value for plasma glucose and insulin is represented as the average increase from the pretreatment value (as 0). Each of the symbols and vertical lines represent the mean ± SE (n=8). *P<0.05; a: Daruma sham-op vs wild-type control, Daruma OCX+T vs wild-type control; b: Daruma sham-op vs Daruma OCX, Daruma OCX+T vs Daruma OCX; c: Daruma OCX vs wild-type control; d: Daruma sham-op vs wild-type control, Daruma OVX vs wild-type control; e; Daruma sham-op vs Daruma OVX+E2, Daruma OVX vs Daruma OVX+E2; f: Daruma OVX+E2 vs wild-type control.

and eventually reached 50–70 *n*g/m*l*, i.e. severe hyperleptinemia, as seen in ICR-Daruma mice. ICR-Daruma mice develop leptin resistance from an early age (4 weeks) [26]. In C57BL/6J-Daruma mice at 4 weeks of age, expression of OB-R mRNA in the liver was substantially lower in both sexes than in wild-type controls. However, the expression of OB-R mRNA in WAT was lower in only male C57BL/6Jj-Daruma mice. These results suggest that male and female C57BL/6J-Daruma mice may develop total and partial leptin resistance, respectively.

It has been reported that impaired glucose metabolism occurs in *ob/ob*, *db/db* [8, 14] or ICR-Daruma mice from a young age [26], but not in *fat/fat*, *tub/tub* [7] or *Ay/a* mice [24]. In C57BL/6J-Daruma mice of both sexes, plasma glucose levels were higher than in wild-type control mice under fasting conditions, but not during *ad libitum* feeding. In addition, plasma insulin levels in C57BL/6J-Daruma mice of both sexes were higher than in wild-type controls. These observations suggest that C57BL/6J-Daruma mice might acquire insulin resistance from an early age. This possibility is supported by the fact that, relative to wild-type controls, both sexes showed decreased expression of mRNAs for 11 genes in WAT and 4 genes related to insulin resistance as early as 4 weeks of age. In addition, C57BL/6J-Daruma mice of both sexes showed impaired responses to exogenous administration of insulin or glucose.

However, under *ad libitum* food intake, although both sexes (especially males) showed hyperinsulinemia, there were no significant differences in plasma glucose levels relative to control mice. In addition, C57BL/6J-Daruma mice of both sexes were free of diabetes until 53 weeks of age. These results suggest that glucose metabolism in C57BL/6J-Daruma mice may be compensated by hyperinsulinemia, induced by a decrease of insulin sensitivity through reduced expression of genes involved in insulin receptor signaling (IRS-1 and IRS-2). However, the rate of diabetes onset in C57BL/6J-Daruma mice was a lot different from that in ICR-Daruma mice. C57BL/6J mice with diet-induced obesity (DIO) show exaggerated insulin sensitivity and glucose tolerance, with obesity and hyperinsulinemia, but do not develop overt diabetes [2, 30]. Therefore, the fact that C57BL/6J-Daruma mice do not develop diabetes may be a characteristic inherited from C57BL/6J mice.

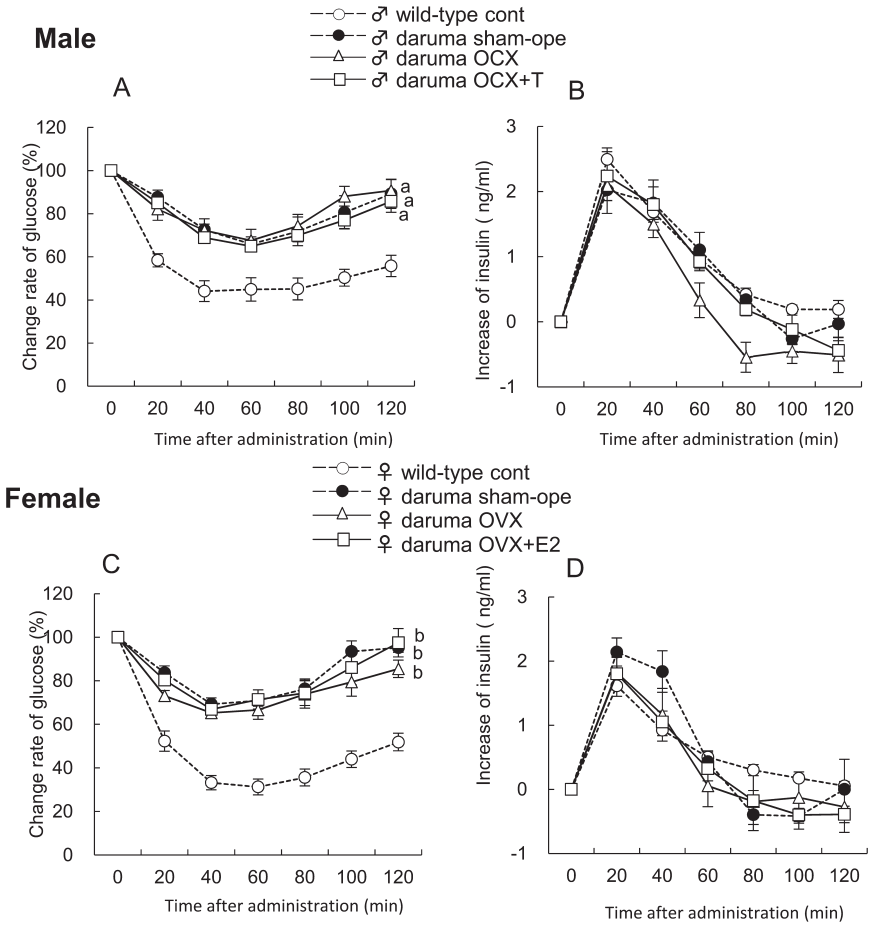

Fig. 6. Comparison of insulin tolerance test data between wild-type control mice, and female and male C57BL/6J-Daruma mice subjected to a sham operation or gonadectomy with or without steroid supplementation. Plasma glucose levels are represented as values relative to the pretreatment value (as 100%). Plasma insulin levels are represented as average increases or decreases from the pretreatment value (as 0). Each of the symbols and vertical lines represent the mean ± SE (n=8). *P<0.05; a: Daruma sham-op, Daruma OCX, Daruma OCX+T vs wild-type control; b: Daruma sham-op, Daruma OVX, Daruma OVX+E2 vs wild-type control.

To examine the possibility that the difference in sex steroid hormone levels in male and female C57BL/6J-Daruma mice may be related to the difference in plasma insulin levels between the sexes, we subjected orchidectomized or ovariectomized C57BL/6J-Daruma mice to chronic administration of testosterone or estradiol.

Orchiectomy in male C57BL/6J-Daruma mice caused a slight blunting of body weight gain and completely suppressed the increase in the plasma level of insulin during growth. On the other hand, testosterone supplementation in orchidectomized C57BL/6J-Daruma mice did not alter the growth curve or plasma insulin levels relative to C57BL/6J-Daruma mice that had been subjected to a sham operation. These results suggest that testosterone may be one of the factors involved in hyperinsulinemia in male C57BL/6J-Daruma mice.

In female C57BL/6J-Daruma mice, neither ovariectomy nor ovariectomy + estrogen supplementation caused hyperinsulinemia, unlike the 40 *ng*/m*l* level seen in male C57BL/6J-Daruma mice. Ovariectomy did not lead to blunting of body weight gain or suppression of the plasma insulin level, and in fact, both showed a slight increase. In addition, chronic administration of estradiol after ovariectomy led to a decrease of body weight and plasma insulin levels relative to C57BL/6J-Daruma mice that had undergone a sham operation. These results are in agreement with a study in which normal female rats were subjected to the same treatment [23]. Therefore, estrogen may not be involved in the insulin secretion and obesity seen in C57BL/6J-Daruma mice.

Glucose tolerance in orchidectomized C57BL/6J-Daruma mice was improved in comparison with wild-type controls through promotion of glucose-stimulated insulin secretion, whereas glucose tolerance in orchidectomized and testosterone-supplemented Daruma mice was restored to the state seen in mice given a sham operation. In addition, insulin sensitivity in C57BL/6J-Daruma mice showed no difference between those subjected to a sham operation and those subjected to orchiectomy and testosterone supplementation. Muthusamy *et al.* reported that the blood insulin level and expression of the insulin receptor gene in liver, skeletal muscle and adipose tissue were decreased by orchiectomy, but restored by testosterone supplementation (except for expression of mRNA for the insulin receptor gene in adipose tissue) [25]. In addition, in isolated pancreatic islets, testosterone caused a rapid increase of insulin release in the presence of non-stimulatory concentrations of glucose [13]. These results, together with the

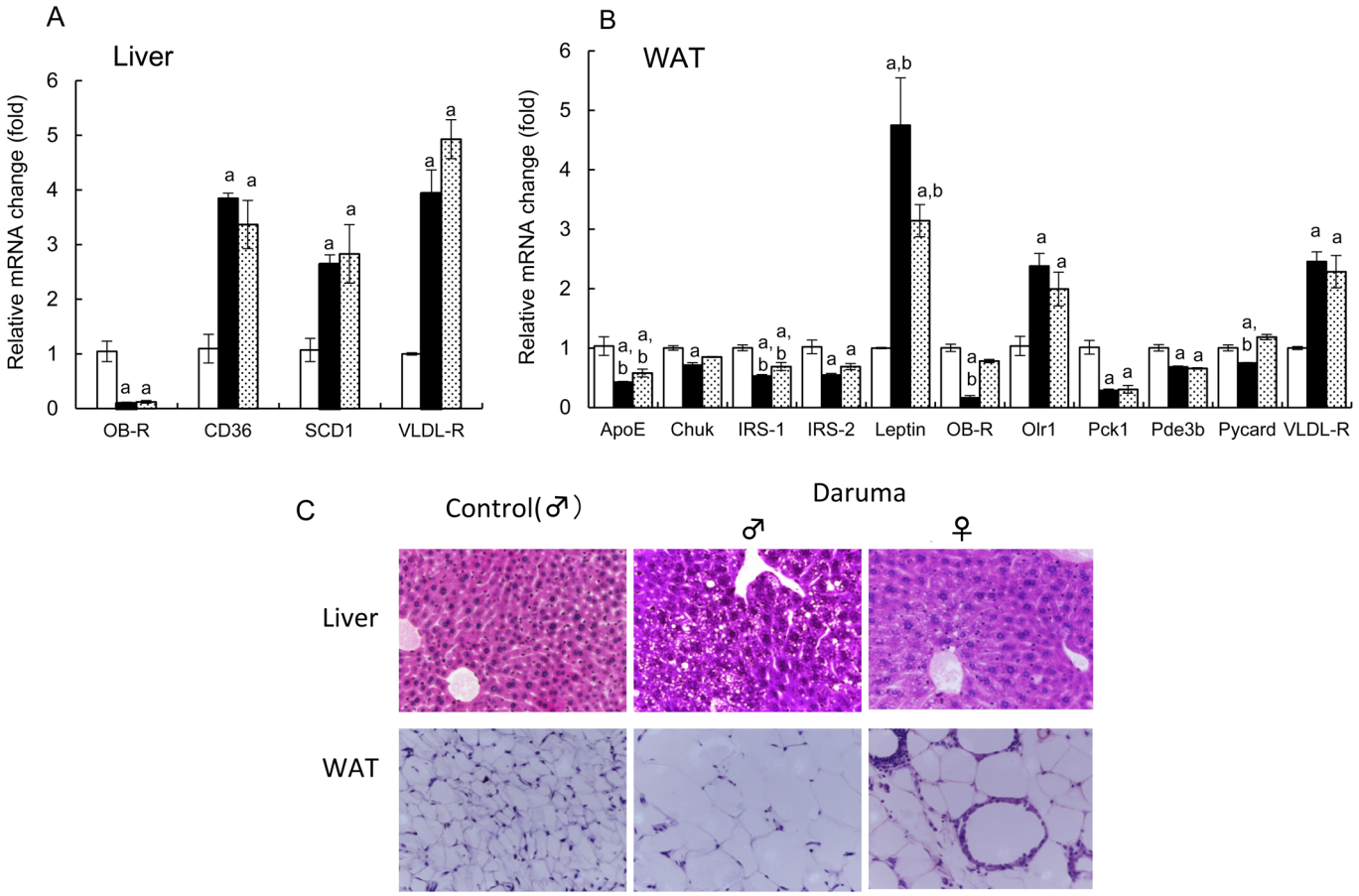

Fig. 7. Expression of mRNAs for genes related to insulin resistance in wild-type and C57BL/6J-Daruma mice. Liver (A) and WAT (B) samples were obtained at 4 weeks of age. White bar, black bar and dotted bar represent wild-type (n=12, 6 male and 6 female), male (n=8) and female (n=8) Daruma mice, respectively. The values are represented relative to those for wild-type mice. As there was no significant difference in the expression of any mRNAs between male and female wild-type mice at 4 weeks of age, averages were obtained by summing all the values for male and female wild-type mice, and each respective value for C57BL/6J-Daruma mice was divided by this average. Each of the symbol and vertical bar represents the mean ± SE. $P<0.05$; a: Daruma mice vs wild-type mice; b: male Daruma mice vs female Daruma mice. (C): Histology in liver and WAT between male and female C57BL/6J Daruma mice at 9 weeks of age. As there was no difference in histological observation between male and female control mice, photos were presented in only male control mice.

present observation that hyperinsulinemia was restored by orchiectomy, whereas testosterone supplementation of orchidectomized Daruma mice again led to hyperinsulinemia suggest that testosterone may affect the difference in plasma insulin levels between male and female C57BL/6J-Daruma mice.

Analysis of PCR array data revealed that expression of mRNAs for CD36, SCD-1 and VLDL-R increased in the liver of both male and female C57BL/6J-Daruma mice at 4 weeks of age. A similar increase in the expression of mRNA for CD36 and SCD-1 in the liver has been reported in ob/ob mice and mice with high fat-induced obesity [5, 15]. In addition, an increase in the expression of mRNA for VLDL-R is involved in fatty liver [16].

Also, in the WAT, expression of mRNAs for many genes related to insulin signaling, such as IRS-1, IRS-2 and Pde3b, is decreased in both male and female C57BL/6J-Daruma mice, suggesting a fundamental disorder in the lipid metabolic system from an early age. On the other hand, the expression of mRNAs for many genes, such as ApoE, Chuk, IRS-1 and 2, OB-R, Pde3b and PYD and Pycard, was significantly decreased in male C57BL/6J-Daruma mice relative to females. On the other hand, there was no change in histological observation in liver and WAT between male and female C57BL/6J Daruma mice at 4 weeks of age (data not shown). However, the lipid droplet accumulation in the liver was observed at 9 weeks of age in male but not female C57BL/6J Daruma mice, and hypertrophic adipocytes in the WAT were observed in both male and female C57BL/6J Daruma mice. Although it is unclear whether these differences in mRNA expression between male and female C57BL/6J-Daruma mice are involved in hyperinsulinemia in males, but not females, further examinations will be required to clarify the relationship between the hyperinsulinemia of male C57BL/6J-Daruma mice and testosterone.

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