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Ontogenetic Characteristics of Enzyme Activities and Plasma Metabolites in C57BL/6J:Jcl Mice Deficient in Insulin Receptor Substrate 2

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We have established an inbred line of mice deficient in insulin receptor substrate 2 (IRS2) that have a C57BL/6J Jcl genetic background (B6J-IRS2$^{-/-}$ mice) as an animal model for typical type 2 diabetes mellitus (DM). We investigated the effect of age and sex on glucose tolerance and insulin resistance and on the activities of enzymes related to lipid metabolism in the liver and skeletal muscle of B6J-IRS2$^{-/-}$ mice. Glucose tolerance tests (GTT), insulin tolerance tests (ITT), and sampling for chemical analysis were performed at ages of 6, 14, and 24 wk. GTT showed that both genders of B6J-IRS2$^{-/-}$ mice had impaired glucose tolerance at the ages of 6 and 14 wk, whereas 24-wk-old female B6J-IRS2$^{-/-}$ mice showed almost glucose tolerance comparable to that of wild-type mice, although 24-wk-old male B6J-IRS2$^{-/-}$ mice still showed impaired glucose tolerance. ITT revealed that both male and female B6J-IRS2$^{-/-}$ mice remained insulin-resistant at all time points. Hepatic lipogenetic enzyme activities were higher in B6J-IRS2$^{-/-}$ mice than in wild-type mice at 6, 14 and 24 wk of age. In addition, plasma glucose, triglyceride, free fatty acid, total cholesterol, and insulin concentrations in B6J-IRS2$^{-/-}$ mice were significantly higher than those in wild-type mice at most time points; plasma triglycerides in 14-wk-old B6J-IRS2$^{-/-}$ mice were lower than those of wild-type mice. These findings suggest that young B6J-IRS2$^{-/-}$ mice are useful as type 2 DM models.

Abbreviations: ACL, ATP citrate lyase; AST, aspartate aminotransferase; DM, diabetes mellitus; FAS, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; GTT, glucose tolerance test; IRI, plasma insulin concentration; IRS, insulin receptor substrate; ITT, insulin tolerance test

Although type 2 diabetes mellitus (DM) is usually an adult-onset disease, it is becoming common in young people.[47,49] At the current increasing rate of onset, it will be one of the world's most common diseases in a few decades and will present public health problems due to an estimated minimum of 500 million cases. This explosion is beginning in the world's 2 most populous countries, India and China, and by 2010, more than 50% of the world's diabetics will be Asian.[20,49] Other populations undergoing increases in the incidence of type 2 DM in recent years include Japanese, aboriginal Australians, Hispanic Americans, and Afro-Americans.[9,48,49]

Human type 2 DM is characterized by peripheral insulin resistance and defective insulin secretion.[1,16,17,29,32] Type 2 DM is associated with disorders of insulin receptor substrates (IRSs), which mediate pleiotropic signals initiated by receptors for insulin and other cytokines.[26] IRS1-deficient mice are growth-retarded and show skeletal muscle insulin resistance[37] but do not develop diabetes because the hyperinsulinemia associated with the β cell hyperplasia in these mice effectively compensates for the insulin-resistant state.[3,30,38,40,46] In contrast, IRS2-deficient (IRS2$^{-/-}$) mice develop diabetes, presumably due to inadequate β cell proliferation combined with insulin resistance.[19,44,45] Another particularly noteworthy feature of IRS2$^{-/-}$ mice is that they exhibit increased adiposity associated with hyperleptinemia, which is involved in the insulin resistance of these mice.[7,39] In fact, the insulin resistance in IRS2$^{-/-}$ mice is ameliorated, at least in part, by reducing the adiposity.[34] Therefore, the analysis of IRS2-deficient mice likely will improve our understanding of the pathophysiology of human type 2 DM.

We backcrossed IRS2-deficient mice (C57BL/6 × CBA hybrid background) generated by Kubota and others[21] with C57BL/6J:Jcl mice to establish an inbred line of IRS2$^{-/-}$ mice, which may provide a tool for studies on medical treatments of type 2 DM. In this study, we examined the effects of the C57BL/6J:Jcl genetic background on the animals' susceptibility to exogenous glucose and insulin stimuli and on the activities of enzymes related to glucose and lipid metabolism in liver and skeletal muscle.

Materials and Methods

Animals. IRS2$^{-/-}$ mice were backcrossed onto the original C57BL/6J:Jcl background (B6J-IRS2$^{-/-}$ mice) for more than 10 generations. B6J-IRS2$^{-/-}$ and wild-type mice were prepared by intercrossing with B6J-IRS-2$^{-/+}$ mice, which were used for vitro fertilization and embryo transfer. IRS-2$^{+/+}$ littermates were used as wild-type controls. The numbers of mice used in this study were: 1) 8, 10, and 8 male B6J-IRS2$^{-/-}$ mice at 6, 14, and 24 wk,

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respectively; 2) 8 male wild-type controls each at 6, 14, and 24 wk; 3) 8 female B6J-IRS2$^{-/-}$ mice each at 6, 14, and 24 wk; and 4) 8, 6, and 8 female wild-type controls at 6, 14, and 24 wk, respectively. All mice were provided with a premium feed for laboratory animals (CA-1, CLEA, Tokyo, Japan) and tap water ad libitum. After weaning, 2 to 3 mice were housed together in each open cage. The animal room was maintained at 24 ± 2 °C, at 55% ± 10% relative humidity, on a 12:12-h light:dark cycle (lights on, 0800 to 2000), and under specific pathogen-free conditions so that *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Pasteurella pneumotropica*, *Salmonella* spp., *Salmonella typhimurium*, dermatophytes, *Pseudomonas aeruginosa*, *Clostridium piliforme*, ectromelia virus, lymphochoriomeningitis virus, mouse hepatitis virus, Sendai virus, cilia-associated respiratory bacillus, mouse parvovirus, mouse adenovirus, mouse encephalomyelitis virus, pneumonia virus of mice, epizootic diarrhea of infant mice virus (rotavirus), mouse cytomegalovirus, reovirus 3, ectoparasites, intestinal protozoa, and pinworms were not detected during this study.

Glucose tolerance tests (GTT), insulin tolerance tests (ITT), and blood sampling for chemical analysis were performed when mice were 6, 14, and 24 wk of age.

This study was approved by the Animal Committee of the Central Institute for Experimental Animals (permit no. 04001) located at Tsukuba and Kumamoto Universities (Japan).

Glucose tolerance test. Mice were fasted for at least 16 h before the study. They then were challenged with an oral glucose dose of 1.0 mg/g body weight. Blood samples were taken from the retroorbital sinus using a heparinized capillary tube at 0, 5, 15, 30, 60, 90, and 120 min after glucose administration, and blood glucose concentrations were measured using an automatic blood glucose meter (Arkray, Kyoto, Japan).

Insulin tolerance test. Two days after the GTT, mice were fasted for at least 3 h before ITT. They were challenged intraperitoneally with human insulin at 0.55 mU/g body weight (human insulin, Eli Lilly Japan, Kobe, Japan). Blood samples were taken from the orbital sinus using a heparinized capillary tube at 0, 20, 40, 60, 80, 100, and 120 min after challenge, and blood glucose concentrations were measured using an automatic blood glucose meter (Arkray).

Chemical analysis. Two days after ITT, blood, liver, and femoral muscle (skeletal muscle) were harvested from mice anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally) and then euthanized between 1100 and 1400; mice had ad libitum access to water and nutritionally complete rodent chow since ITT. Blood was withdrawn from the caudal vena cava into heparinized tubes. Plasma was recovered by centrifugation at 4 °C. Livers and skeletal muscles were excised, quick-frozen in liquid nitrogen, and stored at –80 °C for subsequent analysis.

Plasma glucose concentrations were assayed by the glucose oxidase method.[12] Plasma triglyceride, free fatty acid, and total cholesterol concentrations were measured using commercially available kits (Wako Pure Chemical Industries, Tokyo, Japan). Plasma insulin (IRI) was assayed using immunoreactions according to Arai and others.[2]

Isolation of cytosolic, microsomal, and mitochondrial fractions from the excised tissues[41] and activity assays of hexokinase,[43] glucokinase,[43] pyruvate kinase,[11] lactate dehydrogenase,[18] malate dehydrogenase,[5] aspartate aminotransferase (AST),[31] ATP citrate lyase (ACL),[36] fatty acid synthase (FAS),[10] malic enzyme,[24] phosphenolpyruvate carboxykinase,[15] glucose-6-phosphate dehydrogenase (G6PD),[6] glucose-6-phosphatase,[4] and glutamate dehydrogenase[33] were performed as reported previously.

Histologic analysis. Livers and pancreatic tissues were harvested from 24-wk-old male B6J-IRS2$^{-/-}$ mice with extremely high plasma glucose concentrations. For control samples, livers and pancreatic tissue also were harvested from moderately hyperglycemic B6J-IRS2$^{-/-}$ mice. The tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of pancreatic tissue with islets were stained with hematoxylin and eosin; additional sections were stained with Asan and periodic acid Schiff.

Immunohistochemistry. Sections of liver, fixed in 10% buffered formalin and embedded in paraffin, were mounted on poly-L-lysine-coated glass slides and stained by the avidin–biotin complex method. Sections were pretreated both with 0.3% Triton X-100-containing PBS (pH 7.2) to enhance the penetration of antibodies and with 0.03% H_2O_2 in methanol to block endogenous peroxidase activity. After preincubation with normal goat serum, sections were incubated overnight with rat anti-mouse F4/80 (MCAP497, Serotec, Raleigh, NC), diluted 50- to 100-fold, followed by a 1-h incubation with the streptavidin–peroxidase complex conjugated goat anti rat antibody (Histofine Simplestain Max-PO, Nichirei, Tokyo, Japan). The antigen–antibody reaction was visualized by incubation in 0.05 M Tris-HCl (pH 7.6) containing 0.01% 3,3′-diaminobenzine and 0.001% H_2O_2. Immunostained sections were counterstained with hematoxylin for visualization of nuclei.

Statistical analysis. Differences in body weight, glucose concentration, and enzyme activity between B6J-IRS2$^{-/-}$ and wild-type mice were analyzed by Student *t* test (Sigma Stat 3.0 for Windows, Hulinks, Tokyo, Japan) to compare the mean values. A difference of $P < 0.05$ was considered statistically significant.

Results

Body weights of B6J-IRS2$^{-/-}$ mice. Figure 1 shows the changes in the body weights (obtained just before harvesting of tissues) of B6J-IRS2$^{-/-}$ and wild-type mice at 6, 14, and 24 wk of age. At 6 wk, there was no difference in body weight between B6J-IRS2$^{-/-}$ and wild-type mice of either gender. At 14 and 24 wk, the body weights of female B6J-IRS2$^{-/-}$ mice were increased significantly ($P < 0.05$) compared with those of wild-type mice.

GTT. Figure 2 shows the results of GTT of B6J-IRS2$^{-/-}$ and wild-type mice at 6, 14, and 24 wk. At 6 wk, blood glucose concentrations before and after glucose loading differed significantly ($P < 0.05$) between male wild-type and B6J-IRS2$^{-/-}$ mice. Thereafter, male B6J-IRS2$^{-/-}$ mice continued to maintain strongly impaired ($P < 0.05$ to 0.01) glucose tolerance at 14 and 24 wk. Compared with wild-type mice, 6-wk-old female B6J-IRS2$^{-/-}$ mice also showed significantly ($P < 0.05$) higher glucose concentrations before and after glucose loading. In addition, impaired glucose tolerance in female B6J-IRS2$^{-/-}$ mice was greater at 14 wk than at 6 wk ($P < 0.05$ to 0.01), but glucose tolerance was only minimally impaired in these mice.

ITT. Figure 3 shows the ITT of B6J-IRS2$^{-/-}$ and wild-type mice at 6, 14, and 24 wk. At all time points and for both genders, blood glucose concentrations before insulin injection were already significantly ($P < 0.05$ to 0.01) higher in B6J-IRS2$^{-/-}$ mice than wild-type mice. The glucose concentration-lowering effect of insulin was significantly ($P < 0.05$ to 0.01) impaired in B6J-IRS2$^{-/-}$ mice compared with wild-type mice, suggesting that B6J-IRS2$^{-/-}$ mice show insulin resistance.

Plasma metabolites and enzyme activities. Tables 1 and 2 show

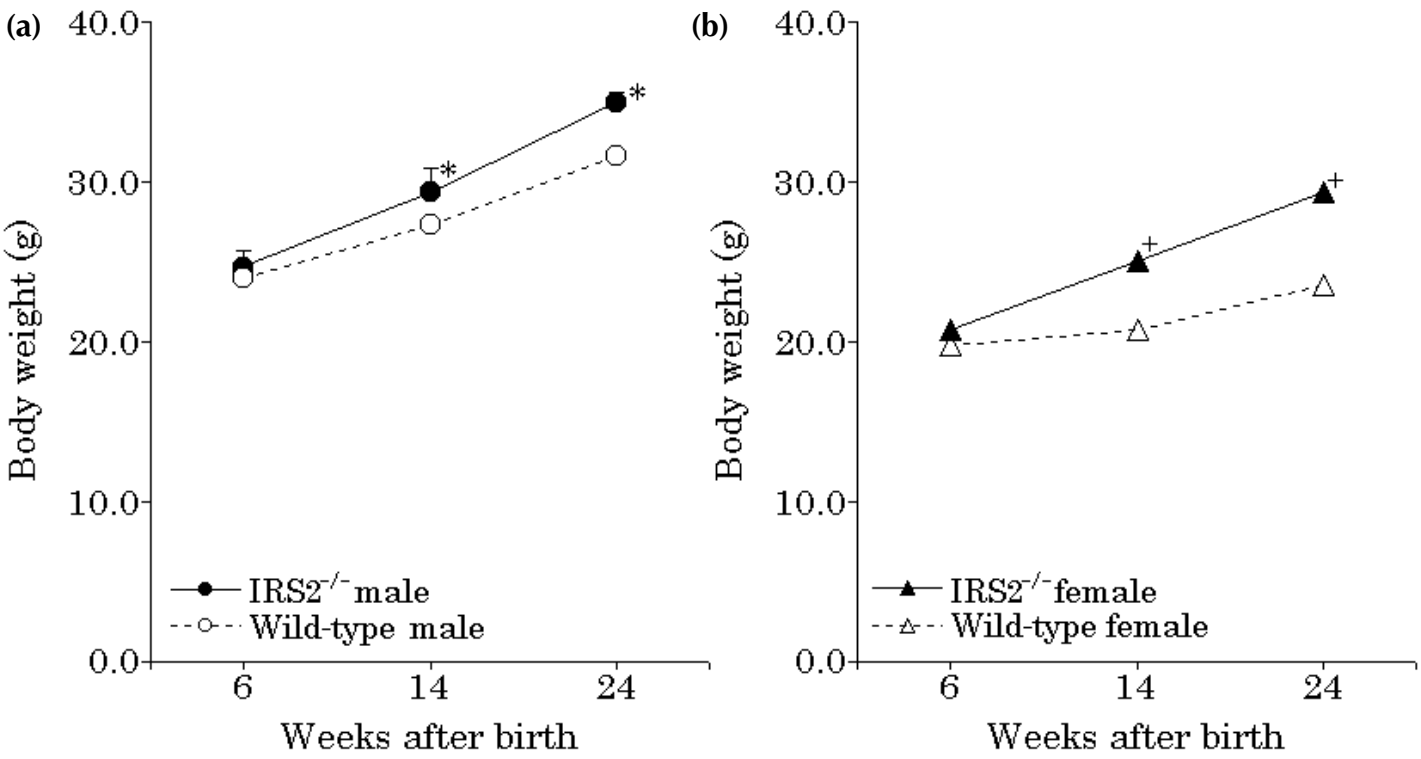

Figure 1. Comparison of body weight between male (a) and female (b) B6J-IRS2$^{-/-}$ and wild-type mice. Data are presented as mean ± standard error. *, $P < 0.05$; +, $P < 0.01$ (Student t test) compared with value for wild-type controls.

the plasma metabolite concentrations and enzyme activities in liver and skeletal muscle in B6J-IRS2$^{-/-}$ and wild-type mice at 6 wk of age. Plasma glucose, triglyceride, free fatty acid, total cholesterol, and IRI concentrations in male B6J-IRS2$^{-/-}$ mice were significantly ($P < 0.05$ to 0.01) higher than those in wild-type mice. In addition, plasma glucose, triglyceride, total cholesterol, and IRI concentrations in female B6J-IRS2$^{-/-}$ mice were significantly ($P < 0.05$ to 0.01) higher than those of wild-type mice.

In the livers of male B6J-IRS2$^{-/-}$ mice, the activities of cytosolic pyruvate kinase, G6PD, ACL, FAS, and malic enzyme activities were significantly ($P < 0.05$ to 0.01) higher than those of wild-type mice. In the livers of female B6J-IRS2$^{-/-}$ mice, cytosolic ACL and malic enzyme activities were higher ($P < 0.05$) than those of wild-type females. In skeletal muscle, cytosolic AST activities in female B6J-IRS2$^{-/-}$ mice were lower ($P < 0.05$) than those of their wild-type counterparts. However, cytosolic ACL activities in female B6J-IRS2$^{-/-}$ mice were higher ($P < 0.05$).

Tables 3 and 4 show the plasma metabolite concentrations and enzyme activities in liver and skeletal muscle in B6J-IRS2$^{-/-}$ and wild-type mice at 14 wk. In both male and female mice, plasma glucose, total cholesterol, and IRI concentrations in B6J-IRS2$^{-/-}$ mice were significantly ($P < 0.05$ to 0.01) higher than those of wild-type mice. However, plasma triglyceride concentrations in male and female B6J-IRS2$^{-/-}$ mice were lower than those of wild-type animals. The results for both males and females showed the same tendency, with cytosolic G6PD, ACL, FAS, and malic enzyme activities higher ($P < 0.05$ to 0.01) in the livers of B6J-IRS2$^{-/-}$ mice than wild-type mice. In skeletal muscle, cytosolic hexokinase activities in female B6J-IRS2$^{-/-}$ mice were lower ($P < 0.05$) than those in wild-type mice.

Tables 5 and 6 show the plasma metabolite concentrations and enzyme activities in liver and skeletal muscle in B6J-IRS2$^{-/-}$ mice and wild type mice at 24 wk. Plasma glucose, triglyceride, free fatty acid, total cholesterol, and IRI concentrations in male B6J-IRS2$^{-/-}$ mice were significantly ($P < 0.05$ to 0.01) higher than those of male wild-type mice. In female B6J-IRS2$^{-/-}$ mice, triglyceride, total cholesterol, and IRI concentrations were higher ($P < 0.05$ to 0.01) than those of wild-type animals.

In liver of male B6J-IRS2$^{-/-}$ mice, activities of cytosolic pyruvate kinase, G6PD, ACL, FAS, and malic enzyme were significantly ($P < 0.05$ to 0.01) higher than those of wild-type mice. In liver of female B6J-IRS2$^{-/-}$ mice, cytosolic G6PD, AST, ACL and FAS activities were higher than those of wild-type females ($P < 0.05$ to 0.01).

Male B6J-IRS2$^{-/-}$ mice with extreme hyperglycemia. Two male B6J-IRS2$^{-/-}$ mice each at the ages of 14 and 24 wk suddenly showed extreme hyperglycemia, similar to that in cases of type 1 diabetes mellitus. Another 2 male B6J-IRS2$^{-/-}$ mice developed extreme hyperglycemia at the age of 11 and 12 wk and died before GTT at 14 wk could be done. The remaining 2 male B6J-IRS2$^{-/-}$ mice showed extreme hyperglycemia at the age of 22 wk, or 7 d before GTT. Extreme hyperglycemia in male B6J-IRS2$^{-/-}$ mice was confirmed by measurement of blood glucose concentrations after thirst and polyuria were observed daily. The 2 male B6J-IRS2$^{-/-}$ mice that became extremely hyperglycemic at the age of 14 wk died just before GTT, but we obtained data from the 2 male B6J-IRS2$^{-/-}$ mice with extreme hyperglycemia and severe polyuria, thirst, and body weight loss at 24 wk (Table 7).

Plasma glucose and free fatty acid concentrations in the extremely hyperglycemic B6J-IRS2$^{-/-}$ mice showed abnormal increases compared with moderately hyperglycemic B6J-IRS2$^{-/-}$ mice ($P < 0.05$). Plasma IRI concentrations in extremely hyperglycemic B6J-IRS2$^{-/-}$ mice were below the detection limit (10 μU/ml). Plasma triglyceride and total cholesterol concentrations in the extremely hyperglycemic B6J-IRS2$^{-/-}$ mice showed similar concentrations

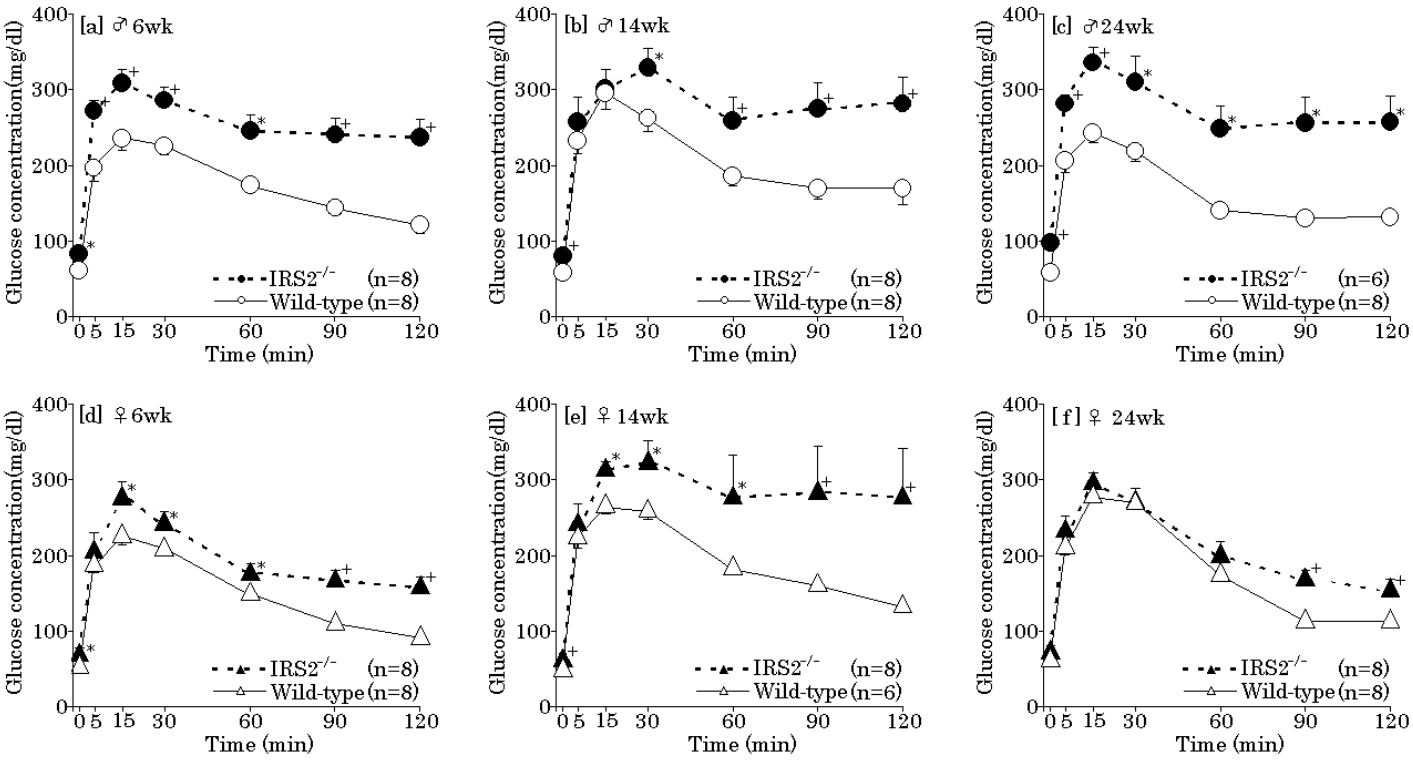

Figure 2. Oral glucose tolerance tests in male (a through c) and female (d through f) B6J-IRS2$^{-/-}$ and wild-type mice at 6 (a and d), 14 (b and e), and 24 (c and e) wk of age. Data are presented as mean ± standard error. *, $P < 0.05$; +, $P < 0.01$ (Student t test) compared with value for wild-type controls.

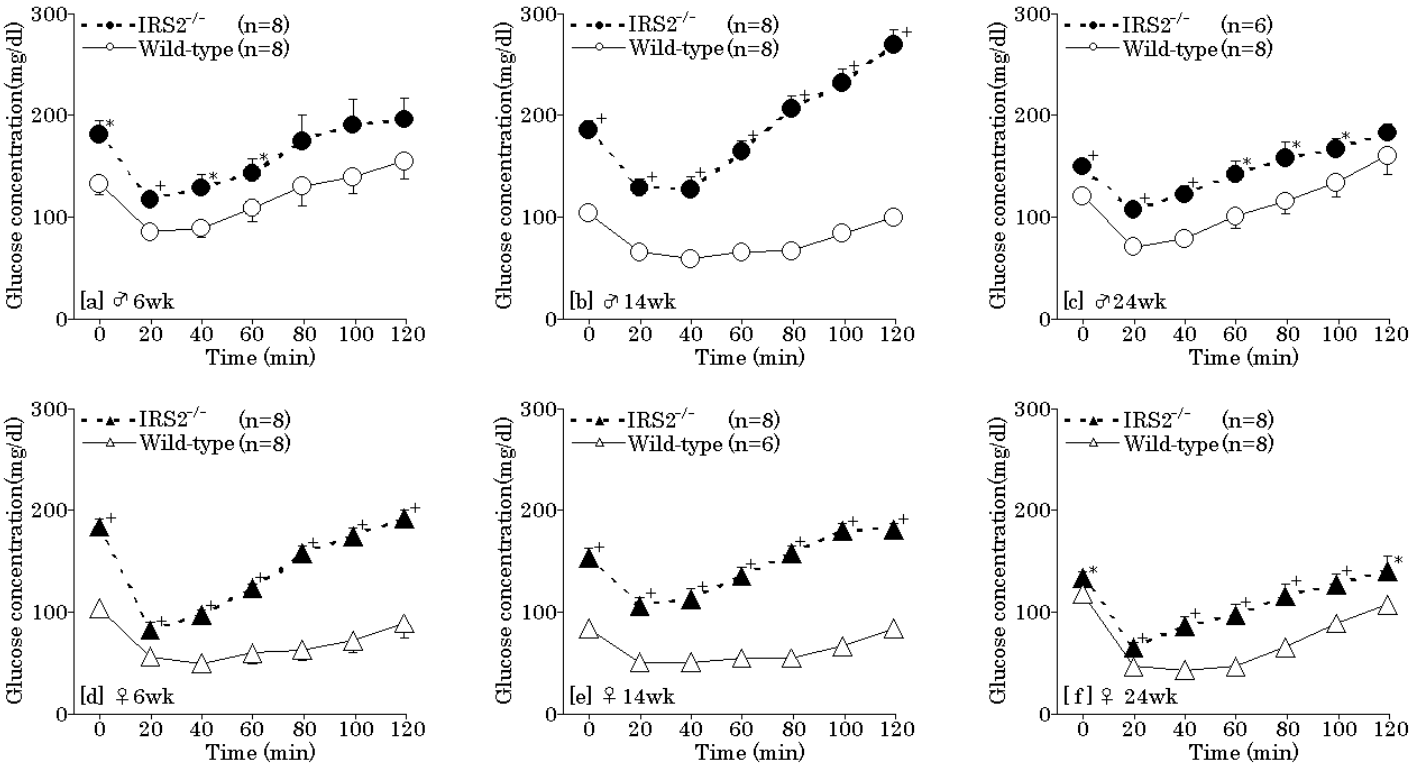

Figure 3. Insulin tolerance tests in male (a through c) and female (d through f) B6J-IRS2$^{-/-}$ and wild-type mice at 6 (a and d), 14 (b and e), and 24 (c and e) wk of age. Data are presented as mean ± standard error. *, $P < 0.05$; +, $P < 0.01$ (Student t test) compared with value for wild-type controls.

Table 1. Plasma metabolite concentrations and hepatic enzyme activities (nmol/min/mg protein) in 6-wk-old B6J-IRS2$^{-/-}$ mice

		Male mice		Female mice	
		Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)	Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)
Plasma					
	Glucose (mg/dl)	152.3 (7.5)	222.8 (23.3)[a]	150.0 (9.8)	209.0 (15.3)[b]
	Triglyceride (mg/dl)	55.4 (2.5)	75.8 (8.4)[a]	55.5 (3.0)	77.3 (9.7)[a]
	Free fatty acid (mEq/l)	0.26 (0.03)	0.49 (0.09)[a]	0.30 (0.06)	0.36 (0.07)
	Total cholesterol (mg/dl)	54.8 (2.6)	88.0 (8.1)[b]	57.8 (5.8)	98.3 (5.4)[b]
	Insulin (μU/ml)	22.3 (2.9)	61.4 (10.5)[b]	26.5 (3.0)	44.4 (8.7)[a]
Liver					
Cytosol					
	Hexokinase	5.6 (0.3)	6.0 (0.6)	5.9 (0.3)	5.5 (0.4)
	Glucokinase	1.4 (0.1)	1.6 (0.2)	1.5 (0.2)	1.8 (0.2)
	Pyruvate kinase	11.1 (1.5)	14.8 (1.4)[a]	15.0 (1.3)	11.5 (1.6)
	Glucose-6-phosphate dehydrogenase	6.0 (0.7)	8.5 (0.8)[a]	6.6 (0.6)	6.9 (0.8)
	Lactate dehydrogenase	1658.6 (75.5)	1633.3 (83.2)	1638.3 (124.9)	1564.5 (81.3)
	Malate dehydrogenase	4339.8 (211.2)	4341.9 (161.8)	4384.4 (195.3)	4359.8 (129.4)
	Aspartate aminotransferase	546.1 (59.6)	577.1 (57.0)	630.3 (25.5)	604.5 (32.4)
	ATP citrate lyase	4.4 (0.4)	5.9 (0.4)[a]	4.2 (0.4)	5.1 (0.3)[a]
	Fatty acid synthase	7.7 (0.6)	10.8 (1.1)[a]	7.7 (0.7)	9.6 (0.9)
	Malic enzyme	10.1 (1.2)	20.1 (2.0)[b]	11.8 (1.6)	17.4 (2.1)[a]
	Phosphenolpyruvate carboxykinase	20.9 (2.2)	22.3 (2.8)	24.2 (2.9)	19.8 (1.6)
Microsomes					
	Glucose-6-phosphatase	424.0 (10.9)	440.1 (21.8)	414.1 (18.6)	397.5 (18.4)
Mitochondria					
	Glutamate dehydrogenase	1264.3 (96.0)	1461.5 (78.5)	1476.0 (212.0)	1544.0 (105.5)
	Malate dehydrogenase	3985.0 (216.4)	4246.8 (348.7)	3957.4 (162.1)	3976.8 (285.3)
	Aspartate aminotransferase	1008.5 (110.6)	981.3 (58.6)	917.1 (55.5)	1019.0 (118.4)

Data are presented as mean (standard error).
[a]$P < 0.05$ (Student t test) versus value for gender-matched wild-type mice.
[b]$P < 0.01$ (Student t test) versus value for gender-matched wild-type mice.

Table 2. Enzyme activities (nmol/min/mg protein) in skeletal muscle of 6-wk-old B6J-IRS2$^{-/-}$ mice

		Male mice		Female mice	
		Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)	Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)
Cytosol					
	Hexokinase	21.5 (1.6)	24.0 (1.9)	21.7 (2.1)	19.8 (0.6)
	Pyruvate kinase	297.1 (10.7)	293.3 (8.0)	289.8 (8.8)	278.4 (8.8)
	Glucose-6-phosphate dehydrogenase	1.3 (0.3)	1.7 (0.1)	1.8 (0.1)	1.5 (0.2)
	Lactate dehydrogenase	5757.6 (191.1)	5873.9 (355.1)	6493.1 (322.4)	5822.5 (248.8)
	Malate dehydrogenase	2406.0 (158.0)	2349.5 (131.7)	2566.1 (131.0)	2245.0 (95.1)
	Aspartate aminotransferase	423.8 (21.7)	461.6 (43.4)	520.6 (35.8)	450.6 (13.0)[a]
	ATP citrate lyase	3.3 (0.3)	2.7 (0.3)	2.3 (0.2)	2.9 (0.2)[a]
	Fatty acid synthase	3.4 (0.3)	3.4 (0.4)	3.2 (0.3)	3.7 (0.2)
	Malic enzyme	8.9 (0.3)	8.8 (0.4)	9.1 (0.6)	9.1 (0.5)

Data are presented as mean (standard error).
[a]$P < 0.05$ (Student t test) versus value for gender-matched wild-type mice.

to those in age- and gender-matched moderately hyperglycemic B6J-IRS2$^{-/-}$ mice.

Table 7 shows the plasma metabolite concentrations and enzyme activities in the livers of extremely hyperglycemic B6J-IRS2$^{-/-}$ mice at 24 wk, in which cytosolic AST and microsomal G6Pase activities were significantly ($P < 0.05$) increased when compared with those of B6J-IRS2$^{-/-}$ mice. In addition, cytosolic hexokinase, pyruvate kinase, LDH, ACL, FAS, and malic enzyme

Table 3. Plasma metabolite concentrations and hepatic enzyme activities (nmol/min/mg protein) in 14-wk-old B6J-IRS2$^{-/-}$ mice

		Male mice		Female mice	
		Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)	Wild-type (n = 6)	IRS2$^{-/-}$ (n = 8)
Plasma					
	Glucose (mg/dl)	163.6 (4.8)	249.5 (23.9)[b]	155.0 (27.6)	218.0 (9.0)[b]
	Triglyceride (mg/dl)	84.8 (8.0)	44.0 (2.9)[b]	59.1 (10.9)	51.9 (4.2)[a]
	Free fatty acid (mEq/l)	0.41 (0.05)	0.31 (0.08)	0.27 (0.05)	0.28 (0.02)
	Total cholesterol (mg/dl)	79.6 (2.6)	95.5 (7.4)[a]	68.1 (12.2)	92.1 (4.8)[a]
	Insulin (μU/ml)	14.3 (2.5)	35.9 (3.9)[b]	11.2 (1.9)	56.3 (14.1)[b]
Liver					
Cytosol					
	Hexokinase	5.0 (0.4)	5.0 (0.5)	5.9 (0.6)	5.7 (0.5)
	Glucokinase	2.0 (0.3)	2.7 (0.4)	2.1 (0.3)	2.9 (0.4)
	Pyruvate kinase	14.1 (0.6)	13.4 (1.1)	12.4 (0.7)	12.4 (0.6)
	Glucose-6-phosphate dehydrogenase	4.8 (0.5)	8.1 (1.0)[b]	4.7 (0.2)	7.3 (0.5)[b]
	Lactate dehydrogenase	1638.6 (70.1)	1575.5 (58.2)	1432.8 (28.6)	1427.6 (65.6)
	Malate dehydrogenase	5089.3 (146.5)	5240.0 (216.8)	5050.2 (227.8)	5341.8 (239.9)
	Aspartate aminotransferase	685.3 (39.4)	697.0 (45.7)	579.5 (57.4)	614.0 (34.9)
	ATP citrate lyase	2.7 (0.2)	6.3 (0.4)[b]	3.2 (0.3)	6.8 (0.5)[b]
	Fatty acid synthase	7.7 (0.5)	10.1 (1.0)[a]	8.2 (0.8)	10.7 (0.7)[a]
	Malic enzyme	9.0 (0.7)	15.9 (1.2)[b]	9.0 (1.0)	15.6 (1.0)[b]
	Phosphenolpyruvate carboxykinase	15.2 (0.8)	16.4 (1.0)	18.4 (1.2)	17.0 (2.1)
Microsomes					
	Glucose-6-phosphatase	466.4 (11.0)	437.9 (26.6)	428.2 (12.5)	417.9 (15.3)
Mitochondria					
	Glutamate dehydrogenase	1624.4 (68.9)	1554.9 (49.9)	1671.7 (74.5)	1559.0 (53.9)
	Malate dehydrogenase	3957.1 (90.0)	3937.8 (164.1)	3800.0 (215.9)	4190.3 (152.2)
	Aspartate aminotransferase	983.1 (28.0)	922.3 (23.5)	1002.8 (55.1)	985.0 (40.0)

Data are presented as mean (standard error).
[a]$P < 0.05$ (Student t test) versus value for gender-matched wild-type mice.
[b]$P < 0.01$ (Student t test) versus value for gender-matched wild-type mice.

Table 4. Enzyme activities (nmol/min/mg protein) in skeletal muscle of 14-wk-old B6J-IRS2$^{-/-}$ mice

		Male mice		Female mice	
		Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)	Wild-type (n = 6)	IRS2$^{-/-}$ (n = 8)
Cytosol					
	Hexokinase	24.2 (1.1)	21.3 (1.1)[a]	26.1 (1.4)	23.1 (1.6)
	Pyruvate kinase	289.0 (5.6)	283.8 (3.7)	283.3 (4.8)	288.6 (12.1)
	Glucose-6-phosphate dehydrogenase	1.4 (0.1)	1.6 (0.3)	1.6 (0.1)	1.5 (0.1)
	Lactate dehydrogenase	6583.9 (249.9)	6331.4 (304.9)	6761.3 (258.0)	6897.3 (134.7)
	Malate dehydrogenase	2688.8 (179.4)	2587.3 (134.1)	2807.2 (152.0)	2631.5 (119.0)
	Aspartate aminotransferase	561.5 (23.7)	545.6 (19.0)	600.8 (24.1)	598.4 (29.6)
	ATP citrate lyase	2.7 (0.1)	2.6 (0.2)	3.6 (0.6)	3.1 (0.2)
	Fatty acid synthase	2.4 (0.2)	2.7 (0.2)	2.9 (0.3)	2.5 (0.2)
	Malic enzyme	8.2 (0.3)	8.1 (0.5)	8.6 (0.6)	7.7 (0.6)

Data are presented as mean (standard error).
[a]$P < 0.05$ (Student t test) versus value for gender-matched wild-type mice.

activities were decreased significantly ($P < 0.05$) in the hyperglycemic mice.

On histopathologic examination, the pancreatic islets of extremely hyperglycemic B6J-IRS2$^{-/-}$ mice were either absent or decreased in size and number compared with those of moderately hyperglycemic B6J-IRS2$^{-/-}$ mice in (Figure 4). The islets of

Table 5. Plasma metabolite concentrations and hepatic enzyme activities (nmol/min/mg protein) in 24-wk-old B6J-IRS2$^{-/-}$ mice

		Male mice		Female mice	
		Wild-type (n = 8)	IRS2$^{-/-}$ (n = 6)	Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)
Plasma					
	Glucose (mg/dl)	165.5 (9.5)	245.8 (12.9)[a]	179.0 (6.7)	191.8 (5.5)
	Triglyceride (mg/dl)	77.3 (4.3)	103.7 (5.6)[b]	79.7 (3.3)	108.0 (5.5)[b]
	Free fatty acid (mEq/l)	0.26 (0.04)	0.50 (0.06)[b]	0.32 (0.06)	0.37 (0.03)
	Total cholesterol (mg/dl)	58.0 (2.6)	80.3 (6.3)[b]	55.7 (6.8)	74.3 (7.1)[a]
	Insulin (μU/ml)	25.8 (2.8)	62.0 (22.0)[b]	20.6 (3.9)	41.9 (8.4)[a]
Liver					
Cytosol					
	Hexokinase	5.4 (0.4)	5.6 (0.4)	5.3 (0.3)	5.1 (0.4)
	Glucokinase	2.0 (0.4)	2.0 (0.3)	1.6 (0.1)	2.2 (0.2)
	Pyruvate kinase	12.1 (0.7)	17.0 (1.4)[b]	12.2 (0.8)	11.4 (1.2)
	Glucose-6-phosphate dehydrogenase	6.1 (0.5)	11.7 (1.3)[b]	6.3 (0.4)	8.8 (0.6)[b]
	Lactate dehydrogenase	1609.0 (86.5)	1755.3 (67.7)	1544.0 (71.2)	1492.9 (76.4)
	Malate dehydrogenase	4880.6 (238.5)	5061.5 (175.7)	4852.3 (306.3)	4582.1 (184.7)
	Aspartate aminotransferase	561.0 (32.2)	513.3 (34.5)	598.3 (32.1)	497.6 (26.3)[a]
	ATP citrate lyase	5.7 (0.5)	9.3 (1.0)[b]	5.4 (0.4)	8.7 (0.9)[b]
	Fatty acid synthase	9.3 (0.6)	12.7 (0.7)[b]	9.2 (0.6)	12.7 (0.4)[b]
	Malic enzyme	19.5 (1.9)	28.8 (1.6)[b]	21.7 (2.8)	26.2 (1.7)
	Phosphenolpyruvate carboxykinase	16.8 (1.8)	18.1 (0.9)	17.5 (1.1)	17.4 (1.2)
Microsomes					
	Glucose-6-phosphatase	424.1 (20.4)	478.5 (34.3)	441.6 (9.9)	456.3 (24.4)
Mitochondria					
	Glutamate dehydrogenase	1627.1 (78.9)	1691.0 (161.0)	1475.0 (40.1)	1609.9 (68.7)
	Malate dehydrogenase	3746.0 (195.0)	3627.0 (92.7)	3488.0 (83.5)	3452.1 (155.3)
	Aspartate aminotransferase	1545.6 (73.1)	528.8 (84.5)	1460.0 (92.1)	1501.4 (93.2)

Data are presented as mean (standard error).
[a]$P < 0.05$ (Student t test) versus value for gender-matched wild-type mice.
[b]$P < 0.01$ (Student t test) versus value for gender-matched wild-type mice.

Table 6. Enzyme activities (nmol/min/mg protein) in skeletal muscle of 24-wk-old B6J-IRS2$^{-/-}$ mice

		Male mice		Female mice	
		Wild-type (n = 8)	IRS2$^{-/-}$ (n = 6)	Wild-type (n = 8)	IRS2$^{-/-}$ (n = 8)
Cytosol					
	Hexokinase	19.9 (1.4)	19.2 (0.9)	19.4 (1.5)	18.9 (0.9)
	Pyruvate kinase	263.9 (26.8)	248.3 (13.8)	267.9 (14.4)	265.6 (10.7)
	Glucose-6-phosphate dehydrogenase	1.2 (0.1)	1.2 (0.2)	1.3 (0.2)	1.3 (0.2)
	Lactate dehydrogenase	6023.1 (235.5)	5630.2 (157.2)	6182.7 (167.8)	5882.4 (271.7)
	Malate dehydrogenase	2543.1 (130.5)	2346.8 (104.9)	2460.3 (58.4)	2531.3 (54.8)
	Aspartate aminotransferase	527.4 (33.8)	497.3 (18.8)	570.0 (21.3)	489.8 (45.5)
	ATP citrate lyase	3.7 (0.4)	4.0 (0.9)	3.8 (0.5)	3.9 (0.5)
	Fatty acid synthase	3.4 (0.2)	3.1 (0.2)	2.9 (0.3)	3.3 (0.6)
	Malic enzyme	8.4 (0.4)	8.8 (0.5)	9.5 (0.4)	10.0 (0.5)

Data are presented as mean (standard error).

extremely hyperglycemic B6J-IRS2$^{-/-}$ mice showed karyorrhexis, cytoplasmic swelling, and partial necrosis. In addition, the liver of 1 extremely hyperglycemic B6J-IRS2$^{-/-}$ mouse showed collagen fibrinoid degeneration (Figure 5 A) and macrophages (Figure 5 B). In addition, the cavitations in the livers of extremely hyperglycemic B6J-IRS2$^{-/-}$ mice at 24 wk appeared to result from fatty degeneration, because PAS staining for glycogen was negative (Figure 5 C).

Table 7. Plasma metabolite concentrations and hepatic enzyme activities (nmol/min/mg protein) in 24-wk-old male B6J-IRS2$^{-/-}$ mice with extreme hyperglycemia

		Wild-type male mice (n = 8)	IRS2$^{-/-}$ male mice (n = 6)	IRS2$^{-/-}$ mice with extreme hyperglycemia (n = 2)
Plasma				
	Glucose (mg/dl)	165.5 (9.5)	245.8 (12.9)	588.5 (28.5)
	Triglyceride (mg/dl)	77.3 (4.3)	103.7 (5.6)	115.5 (27.5)
	Free fatty acid (mEq/l)	0.26 (0.04)	0.50 (0.06)	1.2 (0.3)
	Total cholesterol (mg/dl)	58.0 (2.6)	80.3 (6.3)	76.0 (10.0)
	Insulin (μU/ml)	25.8 (2.8)	62.0 (22.0)	<10
Liver				
Cytosol				
	Hexokinase	5.4 (0.4)	5.6 (0.4)	3.2 (0.8)
	Glucokinase	2.0 (0.4)	2.0 (0.3)	1.4 (0.1)
	Pyruvate kinase	12.1 (0.7)	17.0 (1.4)	7.9 (1.6)
	Glucose-6-phosphate dehydrogenase	6.1 (0.5)	11.7 (1.3)	5.9 (3.6)
	Lactate dehydrogenase	1609.0 (86.5)	1755.3 (67.7)	1130.5 (171.5)
	Malate dehydrogenase	4880.6 (238.5)	5061.5 (175.7)	5300.0 (611.0)
	Aspartate aminotransferase	561.0 (32.2)	513.3 (34.5)	839.5 (202.5)
	ATP citrate lyase	5.7 (0.5)	9.3 (1.0)	3.3 (0.4)
	Fatty acid synthase	9.3 (0.6)	12.7 (0.7)	7.3 (0.05)
	Malic enzyme	19.5 (1.9)	28.8 (1.6)	6.3 (0.8)
	Phosphenolpyruvate carboxykinase	16.8 (1.8)	18.1 (0.9)	19.0 (3.0)
Microsomes				
	Glucose-6-phosphatase	424.1 (20.4)	478.5 (34.3)	995.5 (74.5)
Mitochondria				
	Glutamate dehydrogenase	1627.1 (78.9)	1691.0 (161.0)	1333.0 (43.0)
	Malate dehydrogenase	3746.0 (195.0)	3627.0 (92.7)	4061.0 (77.0)
	Aspartate aminotransferase	1545.6 (73.1)	1528.8 (84.5)	1322.5 (247.5)

Data are presented as mean (standard error).

Discussion

In this study, we conducted detailed metabolic analysis of IRS2$^{-/-}$ mice with a C57BL/6J:Jcl background. We described various chronologic changes in the metabolic profiles of these mice, including changes in glucose tolerance, insulin sensitivity, lipid profiles, and enzymes involved in glucose and lipid metabolism in the skeletal muscle and the liver.

Male and female B6J-IRS2$^{-/-}$ mice both exhibited insulin intolerance, as described in a previous report of IRS-2$^{-/-}$ mice with a mixed genetic background (C57BL/6J × CBA).[21,39] GTT of male B6J-IRS2$^{-/-}$ mice consistently revealed impaired glucose tolerance, but female B6J-IRS2$^{-/-}$ mice only exhibited impaired glucose tolerance at the ages of 6 and 14 wk. In contrast, the glucose excursion curve of 24-wk-old female B6J-IRS2$^{-/-}$ mice was almost indistinguishable from that of wild-type mice, even though ITT revealed persistent insulin resistance in the B6J-IRS2$^{-/-}$ mice (Figure 3). These results indicate gender differences in the glucose tolerance of B6J-IRS2$^{-/-}$ mice.

Kubota and others[21] reported that female IRS-2$^{-/-}$ mice with a mixed background did not show any glucose intolerance throughout the study period. In the current study, however, B6J-IRS2$^{-/-}$ mice exhibited impaired glucose tolerance early during life, until at least 14 wk of age, indicating that genetic background influenced glucose tolerance in female mice more than in male mice. Similar changes in phenotype induced by differences in genetic background occur in obese (db/db) mice. For example, both genders of obese mice with BKS genetic background developed severe early-onset hyperglycemia.[22] In contrast, male—but not female—db/db mice with a C3HeB genetic background (C3HeB-db/db) were diabetes-susceptible.[22] In the current study, the observed gender-associated differences in impaired glucose tolerance of B6J-IRS2$^{-/-}$ mice, with males showing more severe impairment than females, was similar to that in C3HeB-db/db mice. Further, orchectomy inhibited diabetogenesis in male C3HeB-db/db mice, whereas ovariectomy increased the susceptibility of female C3HeB-db/db mice to diabetes.[22] Estrogen, which facilitates hepatic glucose uptake and storage in rodents, provides the most effective means of suppressing excessive hepatic glucose output in susceptible mice.[22] Therefore, the gender-associated differences in the ontogenetic characteristics of B6J-IRS2$^{-/-}$ mice might also result from susceptibility to sex hormone, on account of their C57BL/6J genetic background.

The results of blood chemistry revealed that both male and female B6J-IRS2$^{-/-}$ mice had hyperglycemia and hyperinsulinemia, suggesting the existence of insulin resistance. In addition, the hepatic activities of enzymes involved in glucose and lipid metabolism, such as G6PD, ACL, and FAS, were elevated in B6J-IRS2$^{-/-}$ mice. The significant increases in body weight of

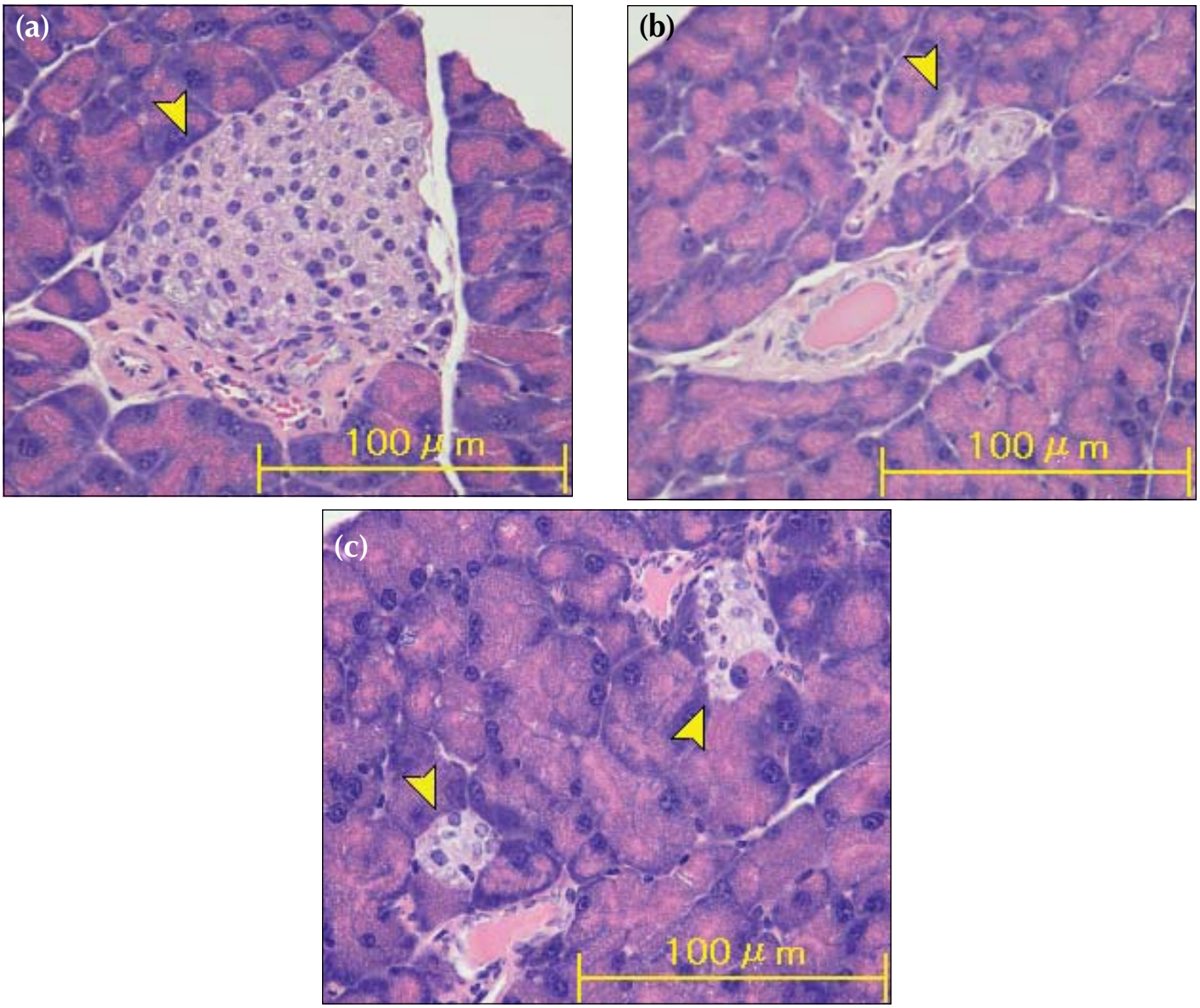

Figure 4. Histopathologic examinations of pancreatic islet cells and liver of B6J-IRS2$^{-/-}$ mice with type 1 diabetes. Pancreatic islets (arrowheads) in a moderately hyperglycemic B6J-IRS2$^{-/-}$ mouse (a) and the 2 B6J-IRS2$^{-/-}$ mice with type 1 diabetes (b, c). Magnification, ×200; scale bars, 100 µm.

14- and 24-wk-old B6J-IRS2$^{-/-}$ may reflect these changes in lipid metabolism. These results are consistent with a report by Tobe and others.[39] Leptin resistance in the hypothalamus is a part of the mechanism underlying the increased enzymatic activities and can be ameliorated by intracerebroventricullar injection of leptin, as reported by Suzuki and others.[35]

Plasma triglyceride and free fatty acid concentrations in B6J-IRS2$^{-/-}$ mice changed in a complex way. The concentrations at 6 wk were elevated compared with those in wild-type mice and then declined over time to even lower concentrations than those of wild-type counterparts. However, by 24 wk, B6J-IRS2$^{-/-}$ mice again had higher triglyceride and free fatty acid concentrations than did wild-type mice. The declines in the triglyceride and free fatty acid concentrations of 14-wk-old B6J-IRS2$^{-/-}$ are quite striking in light of the serial ITT conducted, at the ages of 6, 14, and 24 wk. In most animal models of insulin resistance and in humans, FFA and triglyceride concentrations correlate with the degree of insulin resistance, but 14-wk-old B6J-IRS2$^{-/-}$ mice failed to show such correlation. As judged from the ITT results, insulin resistance in the B6J-IRS2$^{-/-}$ mice was most profound at the age of 14 wk, when their concentrations of triglycerides and free fatty acids were at their lowest during the study period. A similar discrepancy between insulin resistance and plasma triglyceride or free fatty acid concentrations was seen in liver-specific insulin receptor knockout (LIR-KO) mice, as reported by Michael and others.[25] LIR-KO mice exhibited severe insulin resistance in the liver at the age of 2 mo, but their triglyceride and FFA concentrations were diminished markedly.[25] Therefore, the transient decline in triglyceride and free fatty acid concentrations in 14-wk-old B6J-IRS2$^{-/-}$ mice may reflect their increased hepatic insulin resistance.

In this study, we found 2 of 8 24-wk-old male B6J-IRS2$^{-/-}$ mice that showed profound hyperglycemia associated with markedly diminished pancreatic islet size. These extremely hyperglyce-

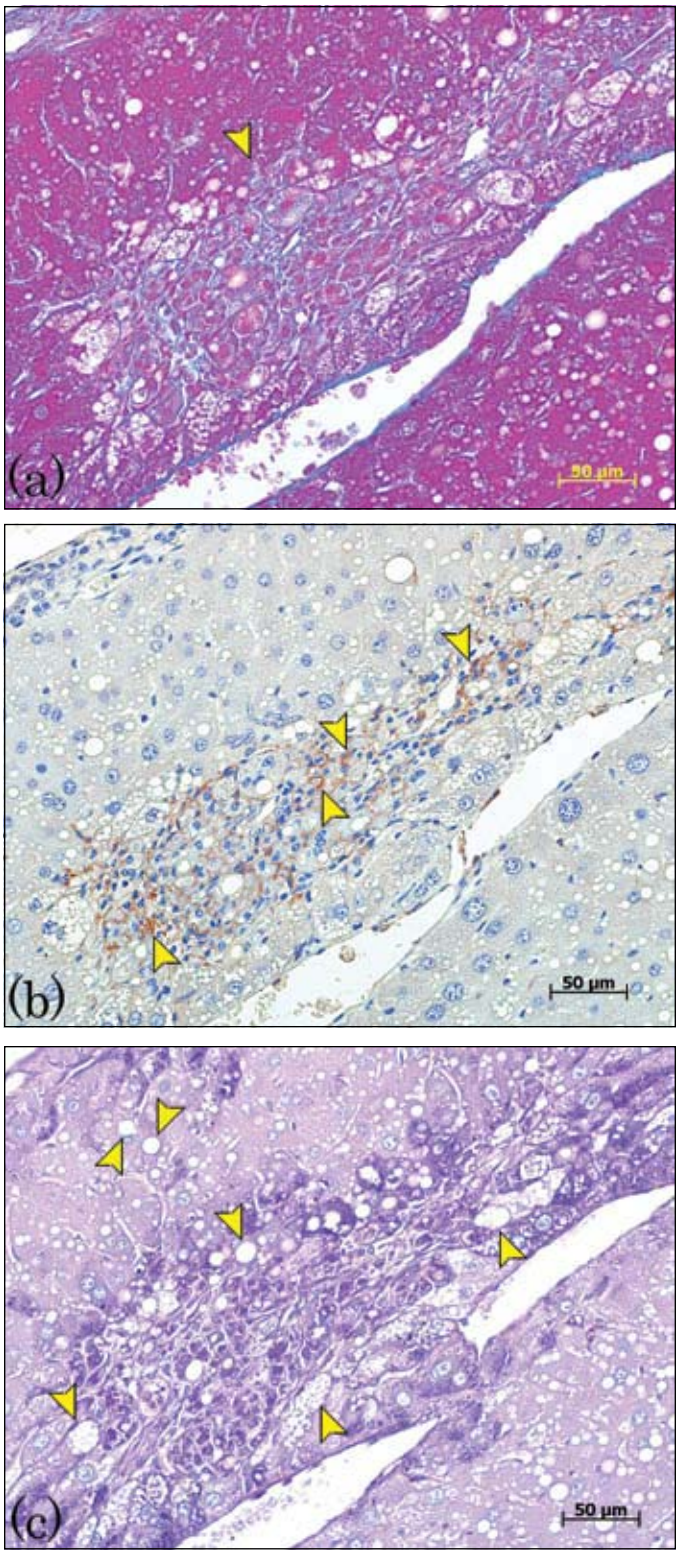

Figure 5. Hepatic tissue from B6J-IRS2$^{-/-}$ mice with type 1 diabetes showing collagen fibrinoid degeneration (a, hematoxylin and eosin stain), macrophages (b, azan stain), and fatty degeneration (c, peridoic acid Schiff stain). Magnification, ×50; scale bars, 50 µm.

mic B6J-IRS2$^{-/-}$ mice had greatly diminished activities of hepatic ACL, FAS, and malic enzyme. In these mice, AST concentrations were elevated, and histochemical analysis of the liver confirmed inflammation. These cases of extreme diabetes resemble the human nonautoimmune fulminant type 1 diabetes reported by Imagawa and others.[13,14] Of the 6 characteristics of this subtype of type 1 diabetes in humans, our extremely hyperglycemic B6J-IRS-2$^{-/-}$ mice exhibited 1) abrupt onset of diabetes and 2) very short duration of diabetic symptoms, such as polyuria, thirst, and body weight loss. However, the mechanisms of development of extreme hyperglycemia associated with diminished islet mass in B6J-IRS2$^{-/-}$ mice are not clear. Recently, Uchida and others[42] reported that nuclear accumulation of p27, a cyclin-dependent kinase inhibitor, in IRS2$^{-/-}$ mice and Lepr$^{-/-}$ mice contributed to the dysfunction of pancreatic beta cells and thus development of diabetes.[42] These mice exhibited markedly diminished islet mass, consistent with our histologic findings in extremely hyperglycemic B6J-IRS2$^{-/-}$ mice. In our male B6J-IRS2$^{-/-}$ mice, cases with high p27 expression or low concentrations of the F-box protein Skp2,[27,28] which inhibits p27, may be more prone to decline in islet mass. However, precise mechanisms of these deleterious patterns of p27 and Skp2 expression are not known. Further, we also noted observed fatty degeneration in the liver of 1 extremely hyperglycemic 24-wk-old B6J-IRS2$^{-/-}$ mouse. The cause of this degeneration might be increased adiposity due to increased activities of lipogenetic enzymes (such as ACL, FAS, and malic enzyme) before the change of glucose tolerance in the B6J-IRS2$^{-/-}$ mouse. We consider that the macrophages noted on histologic exam likely appeared to phagocytize the degraded collagen fibrinoid induced by fatty degeneration. p27 mediates lipid homeostasis in liver,[8] and the degeneration we noted might be related to downregulation of p27 expression.

In conclusion, at the age of 6 wk, B6J-IRS2$^{-/-}$ mice showed profiles compatible with several features of metabolic syndrome, including hyperglycemia, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and high free fatty acid concentration. Therefore even young B6J-IRS2$^{-/-}$ mice are useful animal models for studying type 2 diabetes. Moreover, hyperglycemia and insulin resistance in these mice progressed to their highest levels when the animals were 14 wk old. A small population of male B6J-IRS2$^{-/-}$ mice developed abrupt onset of hyperglycemia associated with markedly diminished islet mass, resembling the features of human nonautoimmune fulminant type 1 diabetes. Our B6J-IRS2$^{-/-}$ mice may also serve as an animal model for studying this subtype of type 1 diabetes.

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