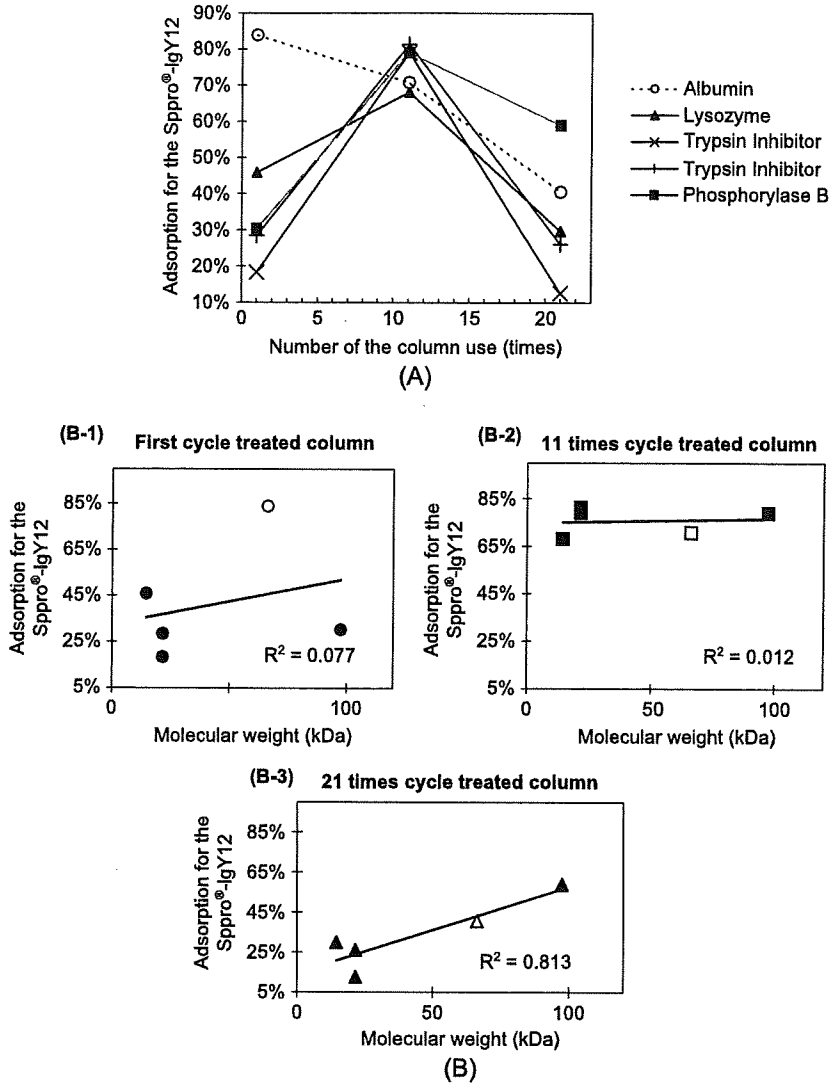

Figure 3. Changes of the adsorption ratio of protein standards for Seppro®-IgY12 column. (A) Relation of the adsorption ratio to the number of times the column was used. (B) Correlation of the adsorption ratio for the column with molecular weight of each protein standard using the column treater one (B-1), 11 (B-2) and 21 (B-3). The open dots show the value for BSA.

direct laser irradiation of the materials could affect the instrument, the positions to be irradiated were the points of the existence of many matrices on a few column materials. Although several peaks existed of less than 70,000 *m/z* in each mass spectrum, the higher molecular weight peaks (7266, 9689, 14,532 and 29,041 *m/z*) appeared in the treated but not in the untreated materials (Fig. 5). After the materials were washed with acetonitrile, the higher molecular weight peaks in the treated materials disappeared (data not shown). Therefore, the compounds attached to the material surface should be hydrophobic high-molecular-weight compounds existing in human plasma.

Two analyses of the column materials surface demonstrated that the hydrophobic high-molecular-weight compounds in plasma adsorbed onto the surface of the affinity column materials and contributed to the changes in the adsorption ability of plasma protein from immunoaffinity into hydrophobic interactions. Howevere, further studies are needed to characterize the exact details of the compounds.

Conclusions

To investigate the ability to remove abundant proteins from plasma by immnoaffinity using the IgY column, FD-LC-MS/MS method was applied to the long-term test of the reproducibility of the column. It was demonstrated that the immunoaffinity column was effective in removing BSA from the protein standards mixture, but, in addition, removing other proteins in the 18.3–45.0% range. The results suggested that the proteins of possible biomarkers could be lost and their quantification made difficult. Moreover, the specific adsorption of BSA in the protein standards mixture and of albumin in the control human plasma samples decreased with an increase in the number of times the column was used with both samples before its use expired. To examine the cause of the functional changes of the immunoaffinity, the correlations between the adsorption ratio for the affinity column and molecular weight of the adsorbed proteins were calculated, and the column materials surface was also investigated by SEM and

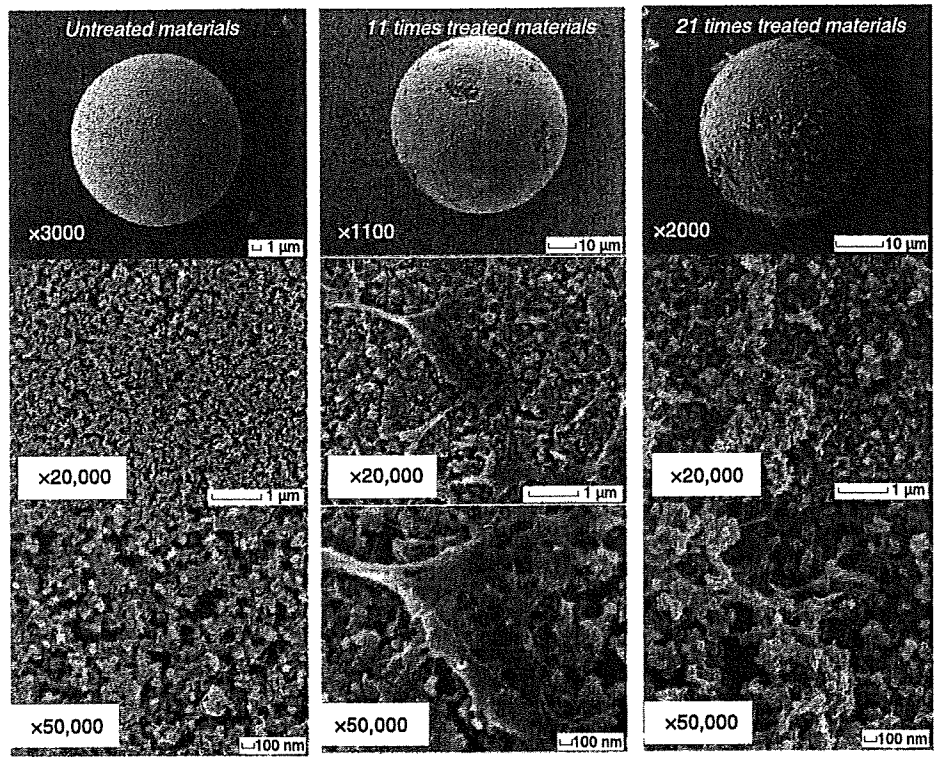

Figure 4. SEM images of the untreated and 11- and 21-times-treated column material surfaces. Magnification in SEM was controlled in a range of × 1100–3000 to show the whole picture of the material.

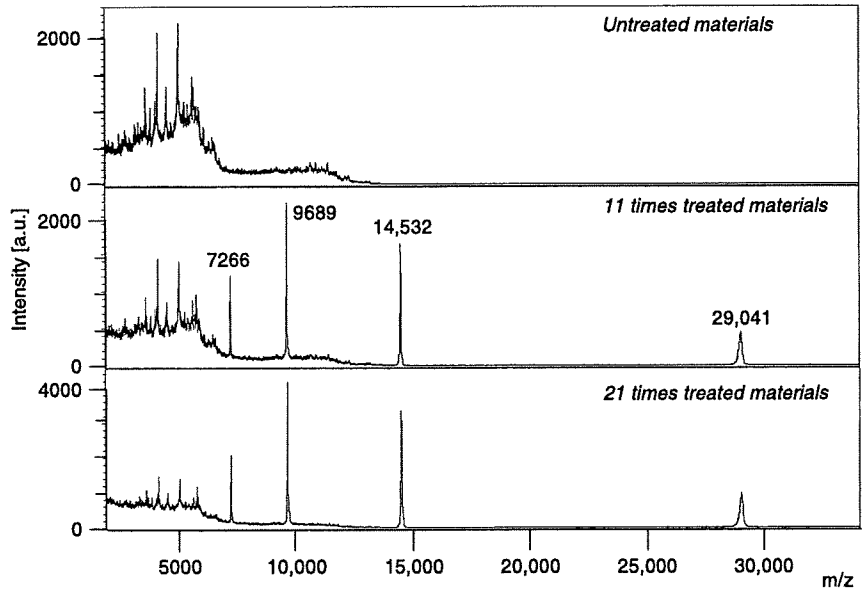

Figure 5. MALDI-TOF-mass spectrum of the untreated and 11- and 21-times-treated column material surface.

MALDI MS analysis. These data demonstrated the attachment of the hydrophobic high-molecular-weight compounds in plasma to the surface, suggesting that on every sample treatment with the affinity column, the adsorption ability of plasma protein changed into hydrophobic interactions. Further studies to characterize the attached compounds are required, and the elucidation of the compounds might lead to the improvement of the affinity column technique and contribute to progress in quantitative plasma proteomics.

Reproducibility is prerequisite for accurate quantitative proteome analysis of clinical samples for biomarker identification and quantification. For this purpose, it is generally essential to

prepare protein samples without high-abundance proteins via specific pre-fractionation techniques to enhance the detection of low-abundance proteins in plasma, and thus, immunoaffinity separation is now chosen as a reliable pre-fractionation method. However, this study indicated that, in quantitative plasma proteomics studies, it is important to keep in mind the risk of not only nonselective loss but also functional changes of the adsorption ability for the immunoaffinity column.

Acknowledgements

We would like to thank Precision System Science Co. Ltd for providing the high-throughput automated proteomic sample processing instrument, Magtration System SA-1, and Bruker Daltonics Japan for performing MALDI MS analysis of the affinity column materials. Part of this work was supported by a MEXT HAITEKU (2004–2008), Grant-in-Aid for Young Scientists (Start-up) and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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Reconsideration of Insulin Signals Induced by Improved Laboratory Animal Diets, Japanese and American Diets, in IRS-2 Deficient Mice

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Key words
- adipocytokines
- obesity
- fatty acid synthesis
- metabolic features
- diabetes
- autoimmunity

Abstract

Current Japanese and American diets and Japanese diet immediately after the War were converted to laboratory animal diets. As a result, current laboratory animal diet (CA-1, CLEA) unexpectedly resembled the diet of Japanese after the War. This is considered to result in an under-evaluation of diabetes research using laboratory animals at present. Therefore, changes in insulin signals caused by current Japanese and American diets were examined using *IRS-2* deficient mice (*Irs2*$^{-/-}$ mice) and mechanisms of aggravation of type 2 diabetes due to modern diets were examined.

Irs2$^{-/-}$ mice at 6 weeks of age were divided into three groups: Japanese diet (Jd) group, American diet (Ad) group and CA-1 diet [regular diet (Rd)] group. Each diet was given to the dams from 7 days before delivery. When the *Irs2*$^{-/-}$ mice reached 6 weeks of age, the glucose tolerance test (GTT), insulin tolerance test (ITT) and organ sampling were performed. The sampled organs and white adipose tissue were used for analysis of RNA, enzyme activity and tissues. In GTT and ITT, the Ad group showed worse glucose tolerance and insulin resistance than the Rd group. Impaired glucose tolerance of the Jd group was the same as that of the Rd group, but insulin resistance was worse than in the Rd group. These results were caused an increase in fat accumulation and adipocytes in the peritoneal cavity by lipogenic enzyme activity in the liver and muscle, and the increase in TNFα of hypertrophic adipocyte origin further aggravated insulin resistance and the increase in resistin also aggravated the impaired glucose tolerance, leading to aggravation of type 2 diabetes. The Japanese and American diets given to the *Irs2*$^{-/-}$ mice, which we developed, showed abnormal findings in some *Irs2*$^{-/-}$ mice but inhibited excessive reactions of insulin signals as diets used in ordinary nutritional management.

received 09.02.2009
first decision 18.05.2009
accepted 27.05.2009

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DOI 10.1055/s-0029-1225352
Published online: 2009
Exp Clin Endocrinol Diabetes

ISSN 0947-7349

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Introduction

Type 2 diabetes mellitus appears to be increasing mainly in the United States, Africa and Asia. In 2000 there were one hundred and fifty million Type 2 diabetic patients, but they are predicted to increase substantially to two hundred and twenty million world-wide in 2010 [37]. Since World War II (WWII), type 2 diabetic patients have increased markedly with dramatic changes of lifestyle in Japan. Typical changes of the lifestyle include the increases in high fat diets, sedentary habits and driving. Especially, the level of fat in modern Japanese diets increased from 20.0 g/day in 1953 to 59.9 g/day in 1995 according to the nation-wide nutrition monitoring survey in Japan. In addition, the Japanese population is predisposed to develop type 2 diabetes due to insufficient insulin secretion in spite of no predisposition to obesity.

Human type 2 DM is characterized by peripheral insulin resistance and defective insulin secretion [11,12]. It is known that type 2 DM is associated with disorders of insulin receptor substrates (IRS), which mediate pleiotropic signals initiated by receptors for insulin and adipokines [7,13,24,26,27] secreted from adipocytes. In IRS family [22,31], *IRS-2* deficient (*Irs2*$^{-/-}$) mice develop diabetes presumably due to inadequate β cell proliferation [14] and increased adiposity [32] combined with insulin resistance. In fact, insulin resistance in *Irs2*$^{-/-}$ mice is ameliorated, at least in part, by reducing the adiposity [28]. Therefore, we thought that *IRS-2* is the central signal in glucose homeostasis. Hashimoto et al. (2006) [5] backcrossed the *IRS-2* deficient mice

Table 1 Conversion from nutrition in human to nutrition contents for laboratory animals.

(a)

	Calorie	Modern American diet[1] Intake weight (kcal/day) 2409.1→462.9[4] Intake weight (g/day)	Relative value (%) 118.7 kcal (%)	Modern Japanese diet[2] Intake weight (kcal/day) 1985.1→384.9 Intake weight (g/day)	Relative value (%) 98.7 kcal (%)	Japanese diet after WWII[3] Intake weight (kcal/day) 390.0 Intake weight (g/day)	Relative value (%) 100.0 kcal (%)
	protein	94.9→18.2	15.8	81.5→15.8	16.4	69.0→13.0	13.3
	fat	92.7→17.8	34.6	59.9→11.6	27.2	20.0→3.8	8.7
	total weight	85.5→16.4	31.9	56.9→11.0	25.8	19.0→3.6	8.3
	SFA	31.3→6.0	11.7	16.0→3.1	7.2	4.3→0.8	1.9
fatty acid	MUFA	35.8→6.9	13.4	20.5→4.0	9.3	6.0→1.1	2.6
	PUFA	18.4→3.5	6.9	20.3→3.9	9.2	8.7→1.6	3.8
	moisture	298.8→57.4	49.6	280.0→54.3	56.4	403.0→76.0	77.9

(b)

	Modern American diet	Modern Japanese diet	Japanese diet after WWII	CA-1
moisture (%)	8.8	10.0	11.1	8.3
crude protein (%)	24.3	22.5	20.2	26.8
crude fat (%)	**15.5**	**10.1**	**3.9**	**5.0**
crude fiber (%)	5.4	5.2	5.2	3.4
crude ash (%)	6.4	6.1	6.2	7.6
NFE (%)	39.6	46.2	53.2	48.9
calorie (kcal/100 g)	395.1	365.0	328.9	347.4
fat energy (%)	35.4	24.8	10.7	–
CP energy (%)	24.6	24.6	24.5	–

[1] USDA data (1994–96)
[2] Nation wide nutrition monitoring survey in Japan (1995)
[3] Nation wide nutrition monitoring survey in Japan (1953)
[4] Conversion from human to mice
SFA: Saturated Fatty Acids
MUFA: Monounsaturated Fatty Acids
PUFA: Polyunsaturated Fatty Acids

(C57BL/6×CBA hybrid background) generated by Kubota et al. (2000) [14] with C57BL/6JJcl mice to establish an inbred line of *IRS-2* deficient mice (*Irs2*$^{-/-}$ mice). As a result, *IRS-2* deficient mice with C57BL/6JJcl genetic background at the age of 6 weeks showed profiles compatible with several features of the metabolic syndrome, including hyperglycemia, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and high FFA when compared with *IRS-2* deficient mice with a C57BL/6×CBA hybrid background.

Diets with excessive fat as a load factor, for example more than 30%, have been fed to various models to show the relation of between type 2 diabetes mellitus and lipid metabolism in many experiments. However, most regular diets fed to type 2 diabetic model mice contain about 5% fat in usual breeding, although one of the factors increasing Japanese type 2 diabetes patients is high fat diets. Therefore, we converted the nutrient content of regular diet for laboratory animals to the human nutrient content on the basis of the nation-wide nutrition monitoring survey in Japan and the National Research Council in the United States. As a result, the nutrient content of CA-1 (CLEA, Tokyo, Japan) as a regular diet became similar to that of the Japanese diet after the WWII. Conversely, the results of converting the nutrient content of modern Japanese and American diets to laboratory animal diets indicated that the fat levels were two or three times higher than that of regular diet such as CA-1. These differences of fat levels suggest the possibility of underestimates of experimental results using various diabetic mice and overlooking of important signals when breeding with regular diets. Therefore, we produced laboratory animal diets that imitated modern Japanese and American diets, and investigated the effects on characteristics of plasma adipokines, metabolites and enzyme activities in *Irs2*$^{-/-}$ mice.

Material and Methods

Animals

IRS-2 deficient mice generated by Kubota et al. (2000) [14] were backcrossed with the original C57BL/6JJcl genetic background (*Irs2*$^{-/-}$ mice) for more than 10 generations. *Irs2*$^{-/-}$ mice were prepared by crossing with *Irs2*$^{-/-}$ mice, which were used for *in vitro* fertilization and embryo transfer. *Irs2*$^{-/-}$ mice were divided to 3 groups, a regular diet (regular diet (Rd) group), Japanese diet (Japanese diet (Jd) group), and American diet (American diet (Ad) group) at the age of 4 weeks. These diets were fed to *Irs2*$^{-/-}$ mice since the embryos were transferred to pseudo pregnant MCH (ICR) mice. *Irs2*$^{-/-}$ mice were housed in Pair Mex II (Osaka Micro system, Osaka, Japan) at the age of 4 weeks, and provided

with regular and Japanese, and American diets as well as tap water *ad libitum*. At the age of 5 weeks, intake rhythm of *Irs2*$^{-/-}$ mice was synchronized with two *Irs2*$^{-/-}$ mice near the average intake weight and pattern in each group. The animal room and specific pathogen-free conditions were the same as previous study [5]. This study was approved by the Animal Committee of the Central Institute for Experimental Animals (Permit No. 06023).

Design of modern Japanese and American diets

Calorie levels for laboratory animal diet (390 kcal/100 g, referenced to NRC, 1995) were based on human Japanese diet after WWII (**Table 1a**). Total calories in human Japanese diet after WWII were taken as a relative value of 100, multiplied by 390 kcal/100 g for both modern Japanese and American diets. Calorie levels in nutrient content of each diet were converted to weight (g)/100 g. As a result of these conversions, these diet contents presented two problems, 1) protein level (percentage of protein calories) was too low, and 2) total levels of protein, fat, and moisture were too high. We thought that these problems might induce inferior growth of infants. These problems were solved by decrease of moisture, increase of protein, and maintenance of fibrous and mineral contents in nutrient combinations. As a result, the practical combination rates of materials and nutrient content are shown in **Table 1b**. The differences between conversion values and practical combination rates of materials were calories and moisture to protect the growth of infants. The nutrient content of regular diet, CA-1, was similar to that of Japanese diet after WWII for laboratory animals. Therefore, we substituted the Japanese diet after WWII for CA-1 as the control in this study.

In vivo Glucose Homeostasis and Chemical analysis

At the age of 6 weeks, glucose tolerance test (GTT), insulin tolerance test (ITT), and harvests of blood, liver, femoral muscles (skeletal muscle), white adipose tissue (WAT), and pancreas for chemical analysis. These protocol and condition were the same as previous study [5].

Plasma glucose concentrations were assayed by the glucose oxidase method [8]. Plasma triglyceride, FFA, and total cholesterol levels were measured using commercially available kits (Wako Pure Chemical Industries, Tokyo, Japan). Plasma insulin was assayed using immunoreactions according to Arai et al. (1989) [1]. Plasma TNFα (eBioscience, California, USA), resistin (AdipoGen, Seoul, Korea), MCP-1 (Pierce Biotechnology, Inc., Rockford, Illinois, USA), and leptin (Ray Biotech, Inc., Norcross, Gergia, USA) were assayed by commercially available ELISA kits.

Isolation of cytosol fractions from the excised tissues [33], and activity assays of hexokinase [34], glucokinase [34], pyruvate kinase [6], aspartate aminotransferase [18], ATP citrate lyase [30], fatty acid synthase [4], and malic enzyme [16] were performed as reported previously.

RNA preparation and Quantitative Real-Time PCR

Total RNA was extracted from liver, skeletal muscle, WAT, and pancreas of *Irs2*$^{-/-}$ mice using TRizol reagent (Invitrogen) following the manufacturer is instructions. RNA (Liver: 500 ng, others: 50–100 ng) was then reverse-transcribed to cDNA using Super Script III RNaseH$^-$ reverse transcriptase (Invtrogen). Real-Time quantitative PCR were carried out with the SYBR Premix Ex Taq™ (TaKaRa) and specific primers for SREBP-1c (Forward: 5′ GGTGTATTTGCTGGCTTGGT 3′ and Reverse: 5′ ACTAATGGGCCCTGATCCTT 3′), PPAR γ 2(Forward: 5′ GGTGAAACTCTGGGAGATTC 3′ and Reverse: 5′ TAATAAGGTGGAGATGCAGG 3′), GLUT2 (Forward: 5′ GGCTAATTTCAGGACTGGTT 3′ and Reverse: 5′ TTTCTTTGCCCTGACTTCCT 3′), GLUT4 (Forward: 5′ TCATTCTTGGACGGT TCCTC 3′ and Reverse: 5′ AGAATCAGCTGCAGGAGAGC 3′) and β actin (Forward: 5′ ACGGGCATTGTGATGGACTC 3′ and Reverse: 5′ GTGGTGGTGAAGCTGTAGCC 3′) according to the manufacturer's instructions. The PCR reactions and detection were performed on a ABI PRISM 7700 using β actin as internal control for normalization purposes.

In addition, reverse transcription (RT)-PCR also was performed to confirm the results of quantitative Real-Time PCR visually followed to PCR conditions according to the manufacturer's instructions. PCR amplification was carried out for 25–35 cycles, consisting of 95 for 30 s, 63.4 °C(SREBP-1c), 59.1 °C(PPARγ2), 64.0 °C(GLUT2), 65.0 °C(GLUT4), or 68.0 °C(ßactin) for 30–40 s, and 72 °C for 5 min in 20 µl of reaction mixture containing 1.5 mM Mg^{2+} and Ex-Tac (TaKaRa, Kyoto).

Histological analysis of liver, WAT, and pancreatic β cells

The liver and WAT were fixed in 10% buffered formalin and embedded in paraffin. Sections of islets were stained with hematoxylin and eosin. Immunohistochemistry of pancreatic β cells was made according to Arai et al. (2008) [2].

Magnetic Resonance Imaging (MRI)

Mice were scanned using 7T Bruker MRI under isoflurane anesthesia. Whole-body was imaged for each mouse in accordance with a fat MRI protocol. Parameters for short T_1-weighted spin-echo pulse sequence were: repetition time=310 ms, echo time=14.7 ms, slice thickness=1.2 mm, field-of-view=2.6×2.6 $(cm)^2$, matrix size=192×192, average=6. A fat image region was evaluated with visual inspection.

Results

Body Weights

Body weights of Rd, Jd and Ad group at 6 wk of age were 20.8±0.4 g (Mean±SEM), 22.7±0.5 g and 22.9±0.5 g each. Japanese and American diet increased the body weights of *Irs2*$^{-/-}$ mice when compared with regular diet ($p<0.05$–0.01).

Glucose tolerance test

Fig. 1a shows the results of GTT in *Irs2*$^{-/-}$ mice fed modern Japanese and American diets for laboratory animals. Blood glucose concentrations before and after glucose loading differed significantly ($p<0.05$) between the Rd group and Ad group, although not between the Rd group and Jd group. Thereafter, the Ad group continued to maintain severly impaired glucose tolerance ($p<0.05$).

Insulin tolerance test

Fig. 1b shows the results of ITT of *Irs2*$^{-/-}$ mice fed modern Japanese and American diets for laboratory animals. Blood glucose concentrations before insulin injection were already significantly higher in the Jd group and Ad group than in the Rd group ($p<0.05$). The glucose concentration-lowering effect of insulin was significantly impaired in the Jd group and Ad group compared with the Rd group ($p<0.05$–0.01), suggesting that the Jd group and Ad group show deterioration of insulin resistance.

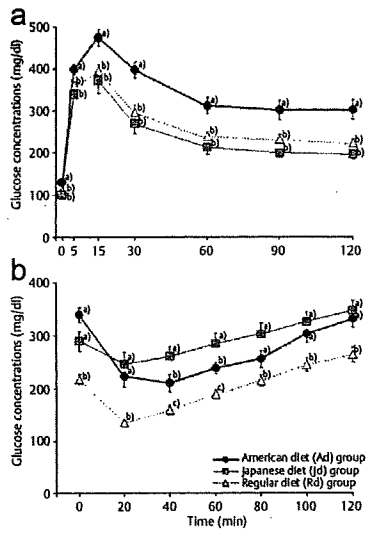

Fig. 1 Effects of modern Japanese and American diets on impaired glucose tolerance and insulin resistance in *Irs2*$^{-/-}$ mice. (a) Glucose tolerance test, (b) Insulin tolerance test Less than $p<0.05$: a) vs. b), b) vs. c), a) vs. c); ANOVA and Tukey's test.

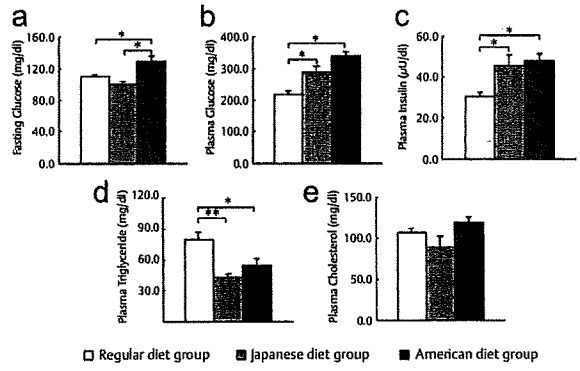

Fig. 2 Effects of modern Japanese and American diets on plasma metabolites in *Irs2*$^{-/-}$ mice. (a) Fasting glucose, (b) plasma glucose, (c) plasma insulin, (d) plasma triglyceride, and (e) plasma cholesterol. Data are presented as mean ± standard error. *: $p<0.05$, **: $p<0.01$ (ANOVA and Tukey's test).

Plasma metabolites

The Ad group showed increased plasma fasting glucose concentration (○ **Fig. 2a**) compared with other groups ($p<0.05$). However, plasma glucose (○ **Fig. 2b**) and insulin concentrations (○ **Fig. 2c**) in the Jd and Ad groups were increased when compared with the Rd group ($p<0.05$). Conversely, plasma triglyceride concentrations (○ **Fig. 2d**) in the Jd and Ad groups were decreased as compared with the Rd group ($p<0.05$). Plasma cholesterol concentration (○ **Fig. 2e**) was the same in the Jd and Ad groups.

Effects of Japanese and American diets on the liver

Expression of SREBP-1c mRNA (○ **Fig. 3a**) in the Ad group was increased compared with the Rd group ($p<0.05$). In addition, expressions of PPAR γ 2 mRNA (○ **Fig. 3b**) and GLUT2 mRNA (○ **Fig. 3c**) in the Ad group were higher than in other groups ($p<0.05$).

Cytosolic glucokinase (○ **Fig. 3d**), pyruvate kinase (○ **Fig. 3e**), and PEPCK activities (○ **Fig. 3f**) were not altered by the differences of diet. Cytosolic fatty acid synthase activities (○ **Fig. 3g**) of the Jd and Ad groups were decreased compared with the Rd group ($p<0.05$). However, ACL activities (○ **Fig. 3h**) of the Ad group increased compared with other groups ($p<0.05$). In addition, malic enzyme (○ **Fig. 3i**) of both the Jd and Ad groups also increased when compared with the Rd group ($p<0.05$). Cytosolic aspartate aminotransferase activities (○ **Fig. 3j**) of the Ad group increased ($p<0.05$), in spite of hepacytes of the Ad group at 6 wk did not differ from the Rd group by histopathologic examination (○ **Fig. 3k**).

Effects of Japanese and American diets on skeletal muscle

Expressions of GLUT4 mRNA (○ **Fig. 4a**) in the Jd and Ad groups were lower than that in the Rd group ($p<0.01$).

Cytosolic hexokinase (○ **Fig. 4b**), glucose-6-phosphate dehydrogenase (○ **Fig. 4b**), ATP citrate lyase (○ **Fig. 4e**) and malic enzyme (○ **Fig. 4f**) were not altered by differences in diet. Cytosolic fatty acid synthase (○ **Fig. 4c**) of the Jd group showed higher activity than in the Rd group ($p<0.05$).

Effects of Japanese and American diets on WAT

Expression of GLUT4 mRNA (○ **Fig. 5a**) was not changed in each group. Expression of PPAR γ 2 mRNA (○ **Fig. 5b**) in the Jd and Ad groups was higher than that in the Rd group ($p<0.05$–0.01).

Both the Jd and Ad groups showed increased plasma TNFα concentrations (○ **Fig. 5c**) compared with the Rd group ($p<0.05$). In addition, the Ad group showed increased plasma resistin concentrations (○ **Fig. 5d**) compared with other groups ($p<0.05$). However, plasma MCP-1 concentrations (○ **Fig. 5e**) were not altered. On the other hand, both Jd and Ad groups showed decreased plasma adiponectin concentrations (○ **Fig. 5f**) compared with the Rd group ($p<0.05$). The Ad group showed increased plasma leptin concentrations (○ **Fig. 5g**) compared with the Rd group ($p<0.05$). Both the Jd and Ad groups showed decreased plasma FFA concentrations (○ **Fig. 5h**) compared with the Rd group ($p<0.05$–0.01).

MRI showed the effects of Japanese and American diets on intraperitoneal WAT in *Irs2*$^{-/-}$ mice (○ **Fig. 6a, b**). Peritoneal WAT was accumulated in mice on Japanese and American diets. WAT around the kidney and testes in the Jd and Ad groups increased in proportion to fat contents of diets when compared with the Rd group. In addition, the Jd and Ad groups were corpulent when compared with the Rd group (○ **Fig. 6c**).

Effects of Japanese and American diets on the pancreas

Expression of GLUT2 mRNA (○ **Fig. 7a**) in the Ad group was the lowest among all groups ($p<0.05$). The Jd and Ad groups showed hyperinsulinemia when compared with the Rd group ($p<0.05$). The rates of increases of insulin concentration in each group after glucose load were the same, but insulin in the Am group at 30 min after glucose load was maintained of higher concentrations than that in other groups ($p<0.05$) (○ **Fig. 7b**). On histopathologic examination of Langerhans' islands, insulin secretion was observed in all three groups (○ **Fig. 7c**).

Discussion

Takahashi et al. (1999) reported that fat content in diets causing impaired glucose tolerance in C57BL/6J mice was a calorie ratio exceeding 40% [29]. Thereafter, high fat diets inducing diabetes in mouse strains have fat contents usually exceeding 40% and as

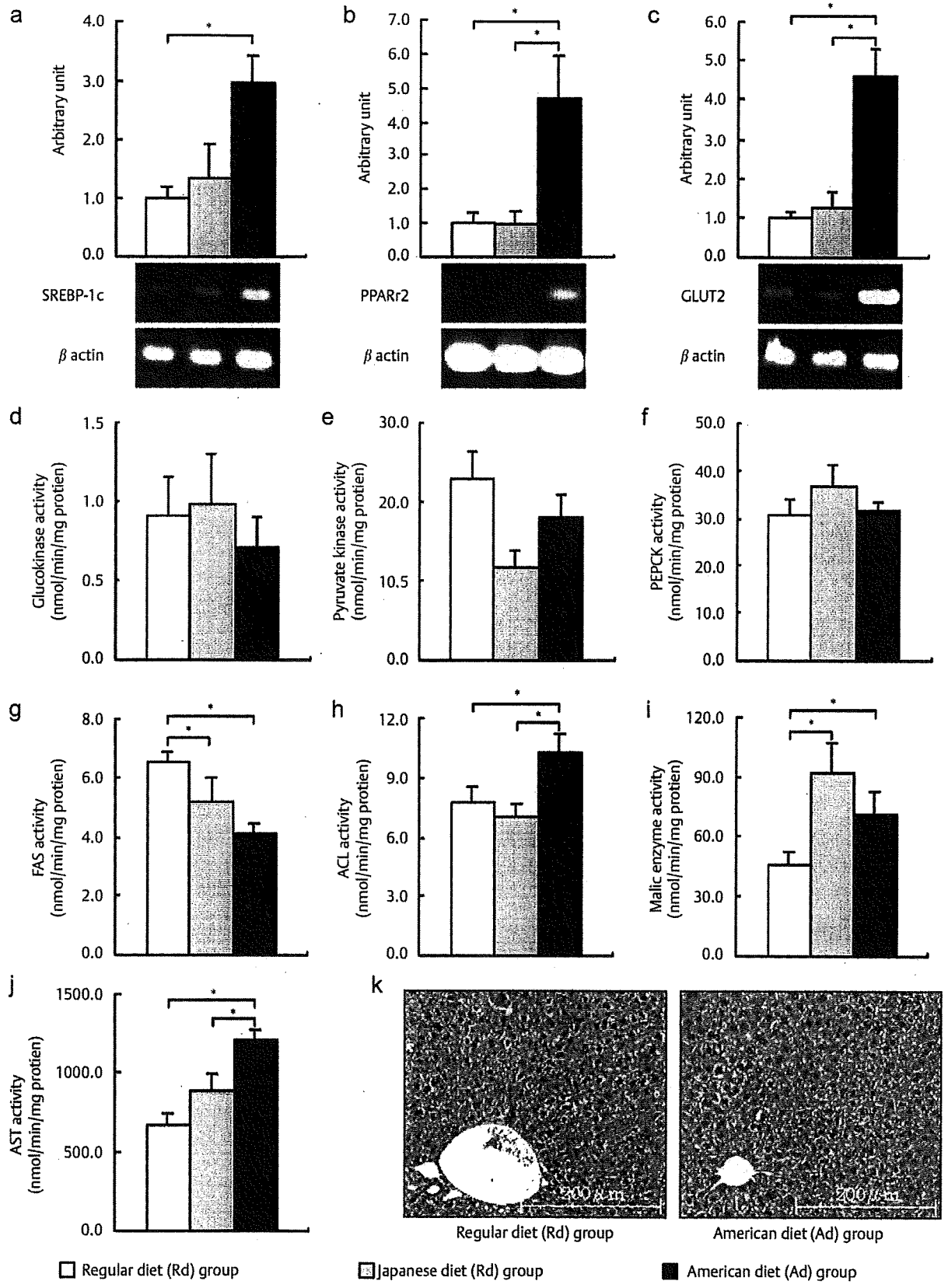

Fig. 3 Effects of modern Japanese and American diets on RNA expression and lipogenic enzymes of liver in *Irs2*$^{-/-}$ mice. mRNA expressions are (**a**) SREBP-1c, (**b**) PPARγ2, and (**c**) GLUT2. Lipogenic enzymes are (**d**) glucokinase, (**e**) pyruvate kinase, (**f**) PEPCK, (**g**) FAS, (**h**) ACL, (**i**) malic enzyme, and (**j**) AST. (**k**) Hepatic histopathologic examinations show regular diet group (Left) and American diet group (Right).

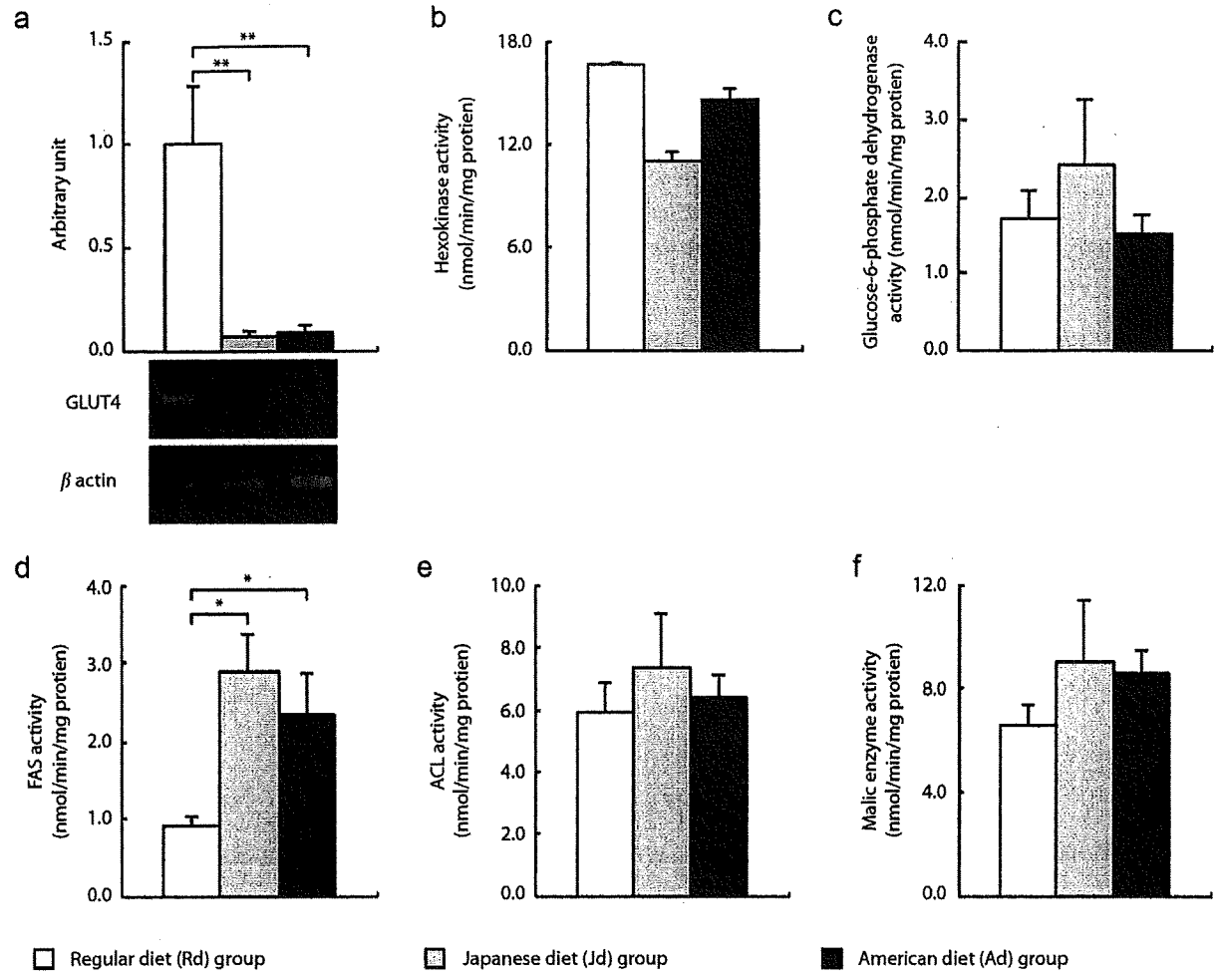

Fig. 4 Effects of modern Japanese and American diets on RNA expression and lipogenic enzymes of skeltal muscle in *Irs2*$^{-/-}$ mice. mRNA expression is (**a**) GLUT4. Lipogenic enzymes are (**b**) hexokinase, (**c**) glucose-6-phosphate dehydrogenase, (**d**) FAS, (**e**) ACL, and (**f**) malic enzyme.

high as 60% [10] in many cases. These diets have much higher fat contents than the usual Japanese diet (Jd) and American diet (Ad) at the moment. The reason for this is that diabetes is difficult to induce in mice when compared with humans and there are major differences in lipid metabolism. However, we have doubts about experiments performed to date using excessively high fat loads. Indeed in Japan in recently years, the people's diet is said to be becoming westernized but continuous daily intake of a diet equivalent to the mouse diet with more than 40% fat content is not usual in Japan or the United States. The effects of the insulin signals obtained from this excessively high fat load are very end-stage and cannot be applied to humans. In research on obesity and diabetes, ob/ob mice [15] and db/db mice are used [35]. Blood sugar levels in these mice are not less than 400 mg/dL, which is the same as that in the NOD mouse [3], a type 1 diabetes mouse model. Therefore, the insulin signals caused by the former high fat diets in strain mice and mutant mice, which were ob/ob and db/db, were highly excessive and terminal disease state. Diabetes in the *Irs2*$^{-/-}$ mouse is mild compared with that in the ob/ob mice [15] and db/db mice [35] and it is a model that approximates latent diabetes. The results of feeding with Jd and Ad to these *Irs2*$^{-/-}$ mice included an increase in intraperitoneal lipids and hypertrophy of adipocytes caused by high expression levels of liver and muscle derived lipogenetic enzyme, hepatic SREBP-1c [10] and fat PPAR-γ2 [9, 17]. The TNFα secreted as a result causes central deterioration of insulin resistance. This condition induces a decrease in glucose uptake by the pancreas and impaired glucose tolerance due to resistin from fat. The difference in impaired glucose tolerance in the Jd and Ad groups appears to be caused by SREBP-1c, PPAR-γ2, GLUT2, ACL and resistin. Resistin is considered to have direct action on impaired glucose tolerance in the Ad group. For FFA, the values in the Jd and Ad groups were lower than those in the Rd group together with those of the triglycerides, which was a highly interesting result. The same phenomenon was observed in a comparison of *Irs2*$^{-/-}$ mice at various ages [5]. At six weeks of age, hypertriglyceridemia and hyper-FFA-emia were more common than in the wild type, but at 14 weeks of age, they were lower in *Irs2*$^{-/-}$ mice than in the wild type. This phenomenon at 14 weeks of age appeared to be caused by deterioration of the disease state at 14 weeks of age [5]. However, where did the triglycerides and FFA that disappeared from the blood go? Since the body weights were significantly increased in the Jd and Ad groups when compared with the Rd group, distribution of visceral fat was observed by MRI, and visceral fat was found to increase in proportion to the degree of the fat load in the diet.

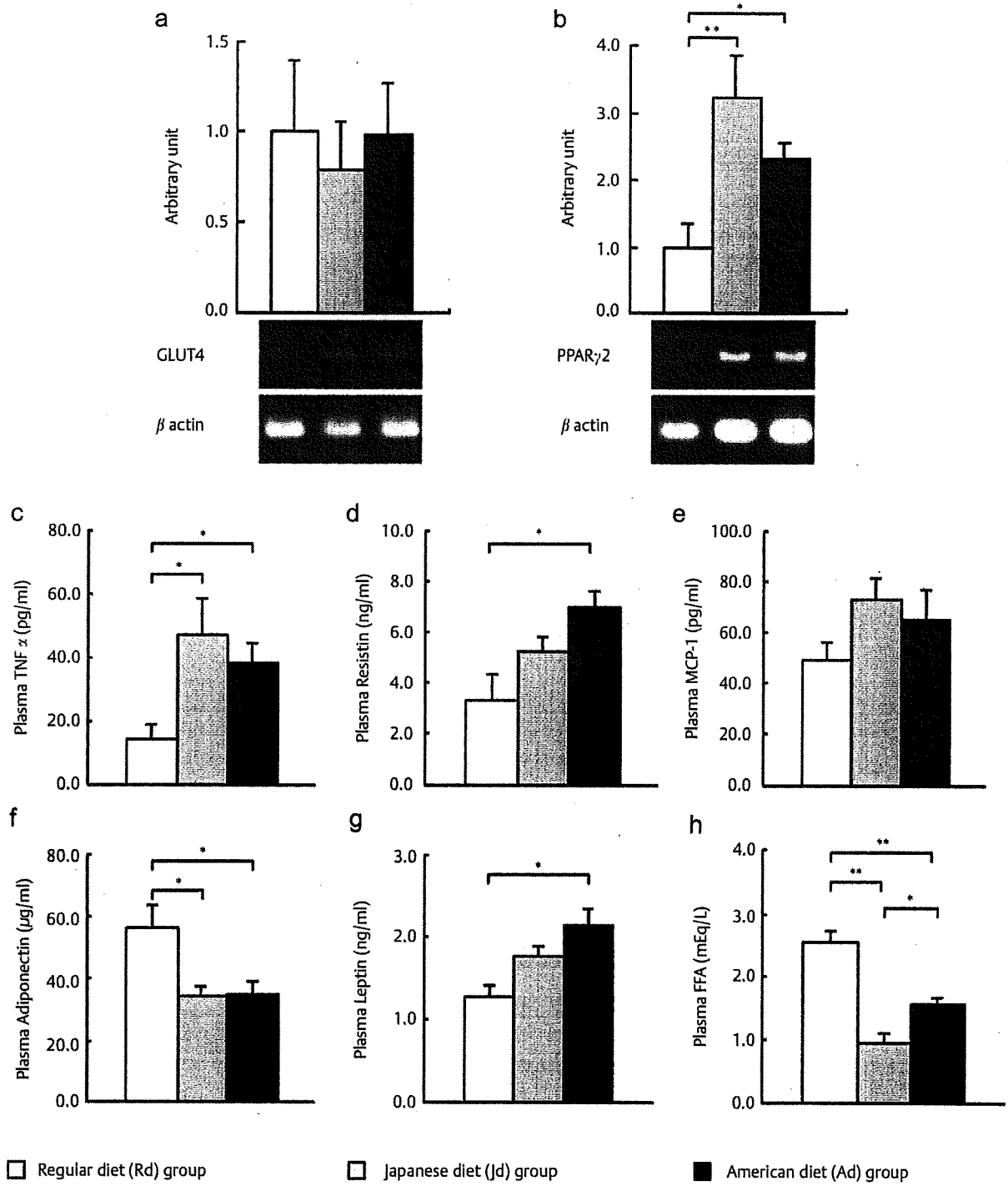

Fig. 5 Effects of modern Japanese and American diets on RNA expression and lipogenic enzyme of white adipose tissue in *Irs2*$^{-/-}$ mice. mRNA expressions for (**a**) GLUT4 and (**b**) PPARγ2. Measured adipokines from white adipose tissue are (**c**) TNFα, (**d**) resistin, (**e**) MCP-1, (**f**) adiponectin, (**g**) leptin, and (**h**) FFA.

The shift of fat from the blood to the peritoneal cavity was a phenomenon observed in *Irs2*$^{-/-}$ mice when a greater fat load was applied. Although this is just a hypothesis, the defect of the *IRS-2* gene related to SREBP-1c is associated with inhibition of fat distribution and fat accumulation is predominantly shifted to the peritoneal cavity. Visceral fat accumulation and hypertrophy of adipocytes were assumed to be caused by expression of PPARγ2 in the fat. Because of phenomena characteristic of *Irs2*$^{-/-}$ mice, it was clear that FFA is not always essential in insulin resistance and TNFα plays the main role. When PPAR-γ2 and SREBP, which hold the key to increased fat and obesity, were compared between the Ad group with the most serious diabetes among the three groups and reports on high lipid loads in mouse strains to date, the expressions of both genes increased and the

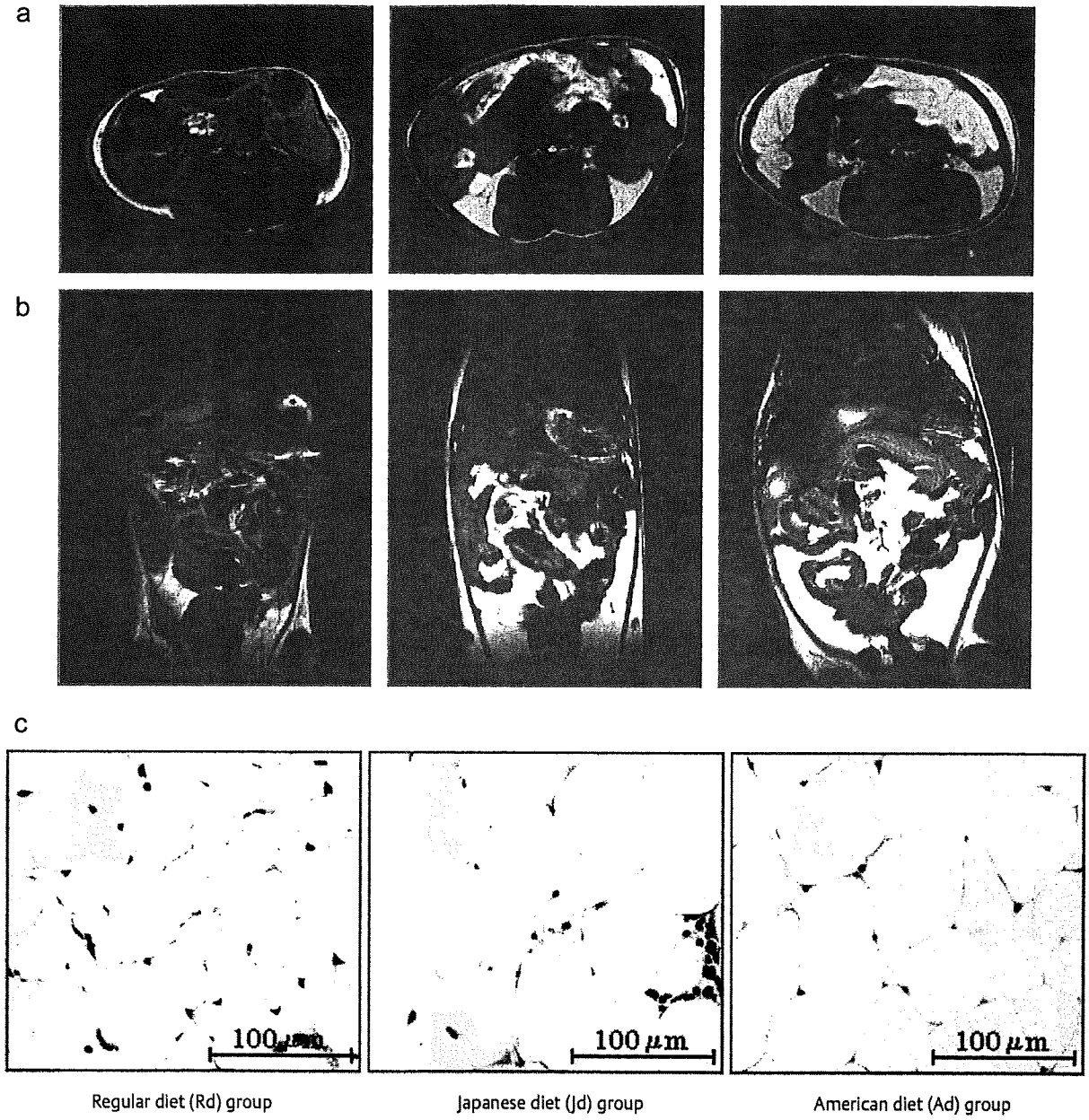

Fig. 6 Effects of modern Japanese and American diets on intraperitoneal white adipose tissues, (a) Axial views, (b) Coronal views of MRI, and (c) Adipocytes in white adipose tissues.

same signal effects were found. The same results were obtained as compared with ob/ob mice [20,23] and db/db [21,36] mice in which diabetes is caused by a different gene. Even when excessive fat loads and excessive diabetes models are not used, it is possible that the same results as reported in the past for SREBP-1c and PPAR-γ2 will be obtained by loading *Irs2*$^{-/-}$ mice with the American diet from the perinatal period. Glucokinase in the liver of a mixed background of wild type, C57BL/6J and DBA/2 mice used by Shiota et al. (2001) showed increased activity on a high fat diet [25]. In a report by Rossmeisl et al. [19], activities of PEPCK in the liver and GLUT4 in the fat of C57BL/6J mice were lowered by a high fat diet. In the present study, they did not show any changes in the Rg, Jd or Ad and the results for hepatic glucokinase and PEPCK and fat GLUT4 were different from those in reports to date. Results of experiments on high fat load with a calorie ratio in excess of 40% show excessive reactions for these parameters. In a past report showing high expression of PPAR-γ2 [9] in the same way as in the present study, fatty liver occurred due to the high fat diet, but in the present study, AST in the Ad group was higher than that for Rg but no signs of fatty liver were observed in a histological examination of the liver. Since young mice at the age of 6 weeks were used in this study, it was inevitable that fatty liver was not observed, but mice are unlikely to develop fatty liver.

The Japanese and American diets given to the *Irs2*$^{-/-}$ mice, which we developed, showed abnormal findings in some *Irs2*$^{-/-}$ mice but inhibited excessive reactions of insulin signals as diets used in ordinary nutritional management compared with high

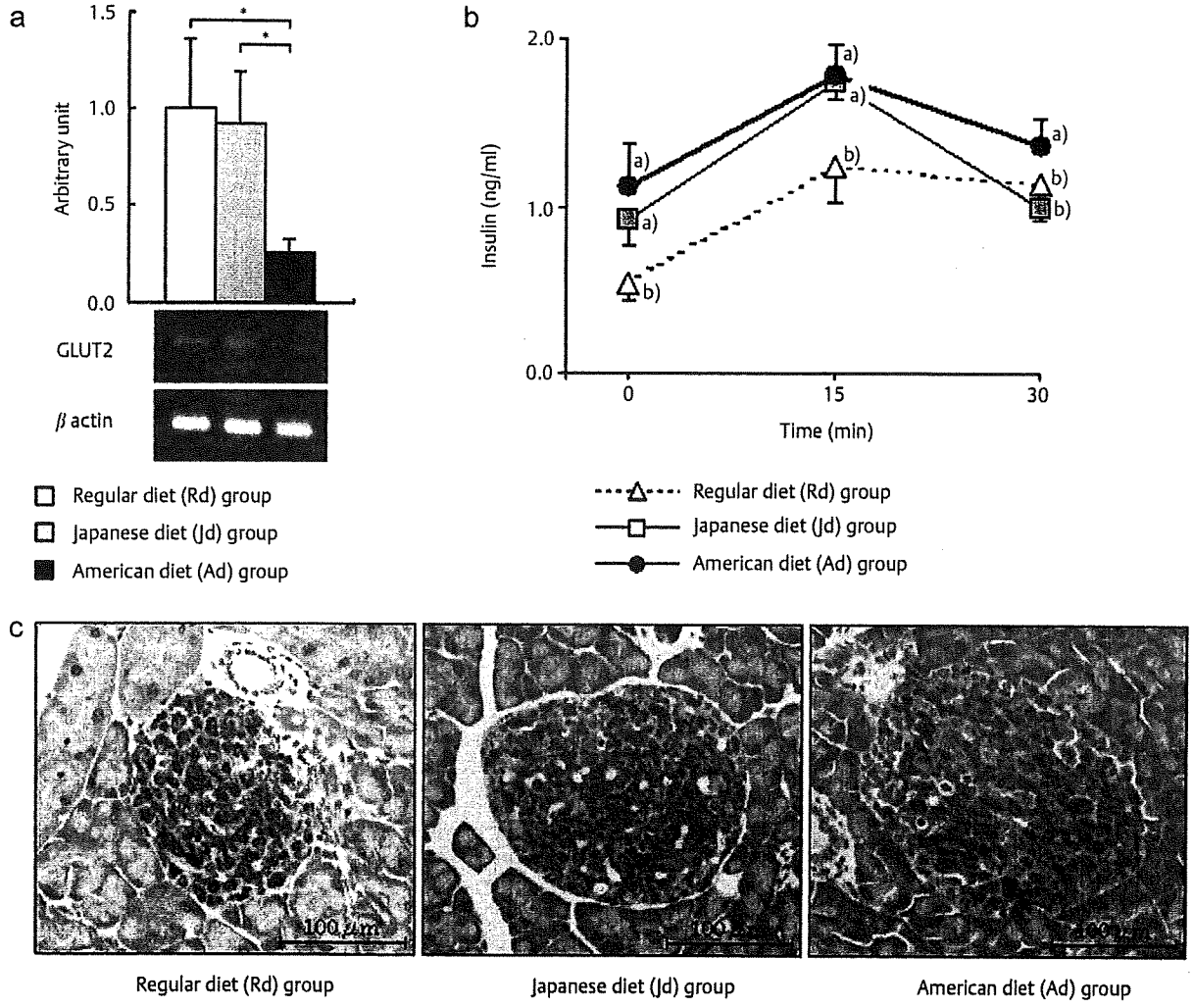

Fig. 7 Effects of modern Japanese and American diets on RNA expression, insulin action and insulin secretion from β cells of pancreas in *Irs2*$^{-/-}$ mice. mRNA expression is **(a)** GLUT4. **(b)** Insulin action for glucose load. **(c)** Immunohistochemistry of pancreatic β cells.

fat diets to date. They each showed characteristic pathophysiology. Therefore, we recommend the use of these diets in ordinary nutritional management since the results obtained in *Irs2*$^{-/-}$ mice given the Japanese diet or American diet in the present study were similar to the results obtained in humans.

Acknowledgements

We thank Tsutomu KAMISAKO, Chie MORI, and Hisako TAKEUCHI for assistence with IVF-ET. husbandry and MRI of mice. This study was partially supported by a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 17200029).

Conflict of interest: None.

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Biochemical and Biophysical Research Communications 382 (2009) 51–56

Contents lists available at ScienceDirect

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Adiponectin suppresses hepatic SREBP1c expression in an AdipoR1/LKB1/AMPK dependent pathway

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ARTICLE INFO

Article history:
Received 18 February 2009
Available online 28 February 2009

Keywords:
Diabetes mellitus
Adipocytokine
Adiponectin
SREBP1c
Fatty acid synthesis
AMPK

ABSTRACT

Adiponectin, one of the insulin-sensitizing adipokines, has been shown to activate fatty acid oxidation in liver and skeletal muscle, thus maintaining insulin sensitivity. However, the precise roles of adiponectin in fatty acid synthesis are poorly understood. Here we show that adiponectin administration acutely suppresses expression of sterol regulatory element-binding protein (SREBP) 1c, the master regulator which controls and upregulates the enzymes involved in fatty acid synthesis, in the liver of $+Lepr^{db}/+Lepr^{db}$ (*db/db*) mouse as well as in cultured hepatocytes. We also show that adiponectin suppresses SREBP1c by AdipoR1, one of the functional receptors for adiponetin, and furthermore that suppressing either AMP-activated protein kinase (AMPK) via its upstream kinase LKB1 deletion cancels the negative effect of adiponectin on SREBP1c expression. These data show that adiponectin suppresses SREBP1c through the AdipoR1/LKB1/AMPK pathway, and suggest a possible role for adiponectin in the regulation of hepatic fatty acid synthesis.

In the pathogenesis of insulin resistance, fat accumulation in liver, or hepatic steatosis, is of great importance. Steatosis can develop as a result of decreased lipid oxidation and increased lipid synthesis. SREBP1c is a critical transcription factor that controls and upregulates the enzymes involved in fatty acid synthesis such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC)-1 in the liver [1–4]. SREBP1c is constitutively upregulated in obese insulin-resistant animal models [5,6], while deletion of SREBP1c in obese model mice ameliorates hepatic steatosis [6]. These data clearly indicate that accelerated lipid synthesis in the liver contributes to the development of hepatic steatosis at least in rodent models, and that SREBP1c could play critical roles in the pathogenesis of fatty liver and the metabolic syndrome.

Adiponectin, whose expression and plasma concentration are inversely correlated with obesity and insulin resistance and type 2 diabetes [7–10], activates AMPK and peroxisome proliferator-activated receptor (PPAR) α, thus increasing fatty acid oxidation in the liver [11–13].

It has previously been reported that adiponectin prevents the development of alcohol-induced steatosis, and also ameliorates fatty liver disease in Lep^{ob}/Lep^{ob} mice [14]. These were attributed to increasing fatty acid oxidation as well as suppressing fatty acid synthesis by adiponectin. However, given the possibilities that improved insulin sensitivity by adiponectin treatment could secondarily affect fatty acid metabolism in the liver, and that central adiponectin signaling interferes with hypothalamic leptin signaling [15,16], it remains unclear whether adiponectin directly suppresses lipid synthesis.

Therefore, the current study is designed to more precisely elucidate the role of adiponectin in hepatic fatty acid synthesis. Here, we show that adiponectin directly suppresses SREBP1c via the AdipoR1/LKB1/AMPK pathway in hepatocytes. The data presented here have revealed an unknown function of adiponectin in the regulation of SREBP1c.

Materials and methods

Generation of recombinant adiponectin. Bacterially expressed murine adiponectin was prepared as described previously [12].

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doi:10.1016/j.bbrc.2009.02.131

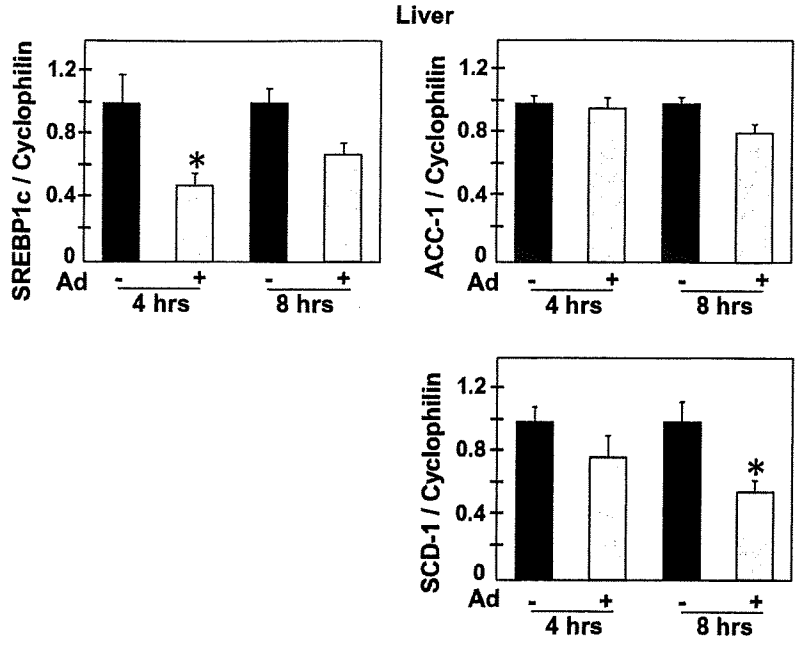

Fig. 1. Intraperitoneal administration of adiponectin acutely suppresses SREBP1c and its downstream molecules. The total RNA was extracted from the liver of *db/db* mice 4 or 8 h after adiponectin administration. Each bar represents the mean ± SE ($n = 9$, $^{*}P < 0.05$).

Animals. Female $+Lepr^{db}/+Lepr^{db}$ (*db/db*) mice were purchased from Japan CLEA. For the immunoblot and gene expression analysis, the mice at the age of 7 weeks were fasted overnight and then injected with 3 μg/g body weight (BW) of recombinant adiponectin intraperitoneally. At 4 or 8 h after injection, their livers were removed. The mice homozygous for a conditional floxed allele of LKB1 (hereafter $LKB1^{lox/lox}$ mice) were generated as described previously [17]. The female $LKB1^{lox/lox}$ mice were subjected to experiments at the age of 7 weeks. The Animal Care Committee of the University of Tokyo approved the animal care and experimental procedures.

Quantitative real-time PCR. The total RNA was extracted from the liver or cultured cells by using an RNeasy kit (QIAGEN). cDNA was prepared by Taqman Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed with ABI Prism by using PCR Master Mix Reagent (Applied Biosystems). Levels of mRNA were normalized to those of cyclophilin mRNA. The sequences of the probes and primers used are as follows; SREBP1c fwd:AAGCTGTCGGGGTAGCGTC, rev: GAGCTGGAGCATGTCTTCAA, probe:ACCACGGAGCCATGGATTGCACATT;cyclophilin fwd: GGTCC TGGCATCTTGTCCAT,rev: CAGTCTTGGCAGTGCAGATAAAA, probe: CTGGACCAAACACAAACGGTTCCCA. The primers and probes of the other genes examined were purchased from Applied Biosystems.

Cells and cell culture. Fao cells were cultured in 6 cm dishes (CORNING) with RPMI1640 medium containing 10% (vol./vol.) fetal bovine serum (GIBCO) and 50 units/ml of penicillin/streptomycin. After equilibration with serum free medium overnight, the cells were incubated with 25 μg/ml of adiponectin.

Transfections and luciferase assays of SREBP1c promoter activity. Luciferase reporter plasmid harboring 2.6 Kbp of 5′-flanking region of mouse SREBP1c exon 1 subcloned to pGL2 basic vector was kindly provided by H. Shimano, Tsukuba University, Tsukuba, Japan. Fao cells placed onto a 24-well plate (CORNING) were transfected with 1 μg of the luciferase reporter plasmid and 0.02 μg of Renilla luciferase plasmid with HSV-TK promoter (phRL-TK, Promega) by using Lipofectamine 2000 (Invitrogen). On the 4th day after overnight starvation, the cells were stimulated with reagents. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacture's protocol.

Generation and infection of adenoviruses. The adenoviruses encoding siAdipoRs were generated according to the manufacture's protocol (TaKaRa Biotechnology). The sequences of target genes were; siAdipoR1, GACGATGCTGAGACCAAAT; siAdipoR2, CCCGACT CTTCTCTAAATTG. An adenovirus encoding shRNA sequence for GFP was used for their control. The adenovirus encoding a dominant negative mutant of AMPKα1 subunit, Cre recombinase and

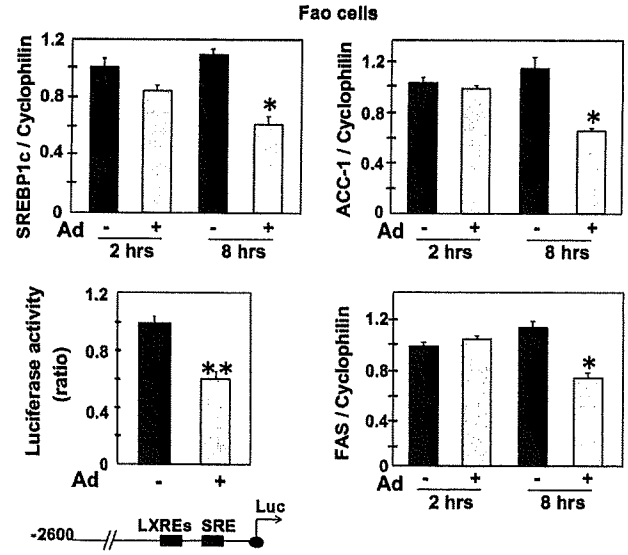

Fig. 2. Adiponectin stimulation acutely suppresses the mRNA expressions involved in fatty acid synthesis in Fao cells. Fao cells are incubated with adiponectin for 2 or 8 h. Each bar represents the mean ± S.E. ($n = 9$, $^{*}P < 0.05$). For luciferase assay, Fao cells transfeced with the reporter plasmid were incubated with adiponectin and 40 pM of insulin. Each bar represents the mean ± SE ($n = 3$, $^{**}P < 0.005$).

LacZ were prepared as previously described [12,17]. The mice were injected with the adenoviruses at a dose of 4×10^9 PFU/gBW for AdipoR knockdown, or at a dose of 0.9×10^{12} PFU/body for LKB1 gene deletion. On the 7th day, the mice were subjected to the experiments.

Immunoprecipitation and immunoblotting. The liver lysates were extracted with the homogenization buffer [18]. A total of 7 mg of liver protein for adipoR1 and 15 mg of liver protein for AdipoR2 were immunoprecipitated with 0.5 μg of the respective antibodies (prepared by IBL Japan). The samples were prepared with Laemmli buffer without boiling. Fao cells were lysed with buffer A [18]. The lysates or the precipitates were subjected to Western blotting by using antibodies for AdipoRs, AMPK (Cell Signaling Technology) and phosphoAMPK (Cell Signaling Technology). The blot was detected by using a chemiluminescence (ECL) system (Roche Molecular Biochemicals).

Statistical analysis. Statistical analysis was performed by 2-sample *t*-test assuming unequal variances or paired 2-sample *t*-test for means. Statistical significance was accepted at $P < 0.05$ unless otherwise indicated.

Results and discussion

Adiponectin suppressed the expression of SREBP1c in the liver of db/db mice

We used *db/db* mice to evaluate the effects of adiponectin on hepatic lipid metabolism. These mice not only show obesity and

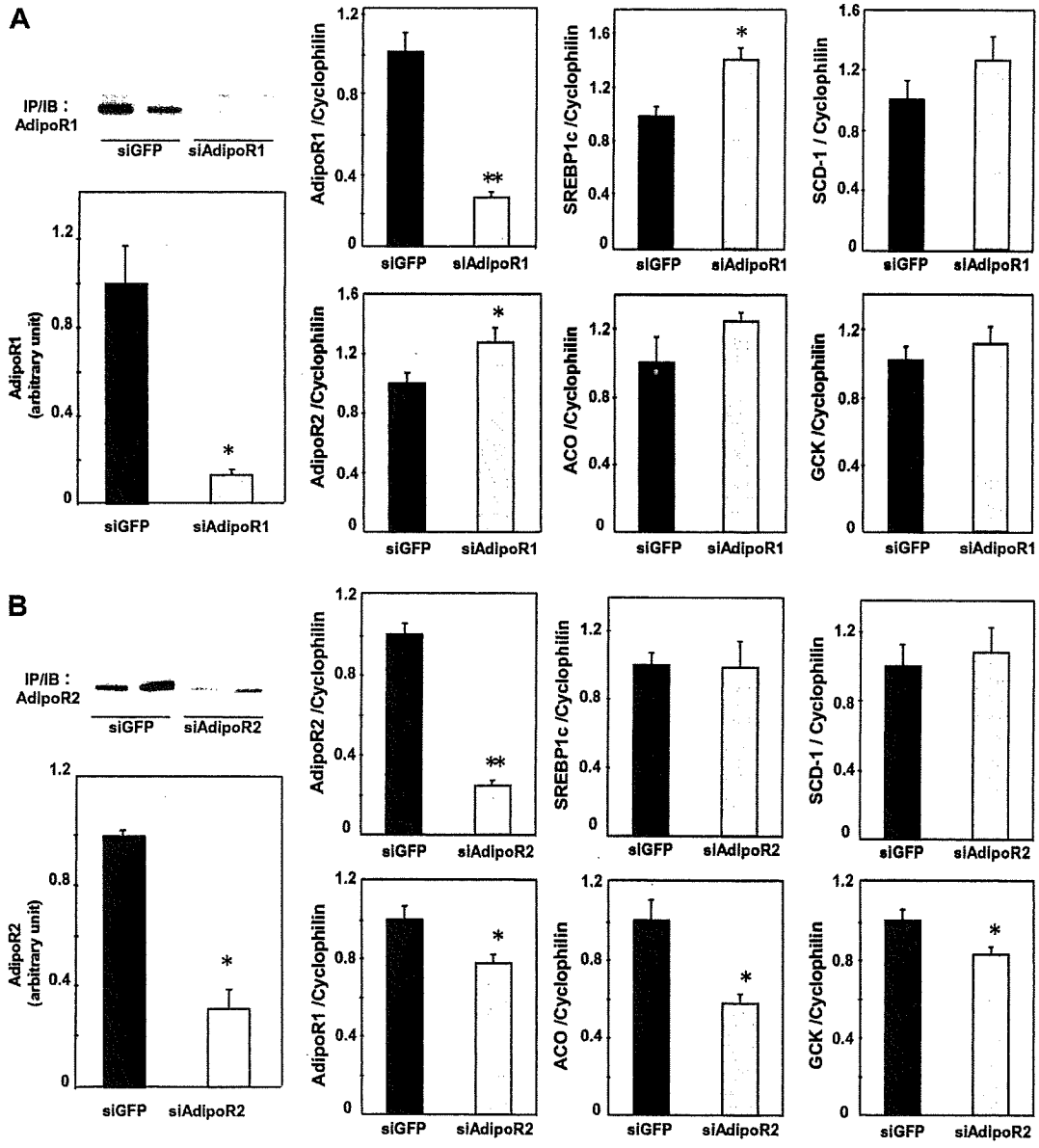

Fig. 3. Adenovirus-mediated gene transfer of siAdipoR1, not siAdipoR2, upregulates SREBP1c expression. The livers were removed from the *db/db* mice on the 7th day after injection of an adenovirus encoding (A) siAdipoR1 or (B) siAdipoR2. The left panels show the representative blot and the results of the quantificational analysis of AdipoRs. Each bar represents the mean ± SE ($n = 4$, $^{*}P < 0.005$). The right panels show the changes in gene expressions in the liver. Each bar represents the mean ± SE (subjects $n = 13$, except for the control group in siAdipoR1 experiment, where $n = 12$, $^{*}P < 0.005$, $^{**}P < 0.0001$).

several characteristics of the metabolic syndrome [19], but also possess some additional advantages; the hyperinsulinemic and hyperglycemic phenotypes make it feasible to observe the direct effects of adiponectin on fatty acid metabolism, apart from the possible secondary changes in the metabolic parameters [1–3,20]. Furthermore, adiponectin could interfere with the central leptin signaling [16], which substantially affects fatty acid metabolism in the liver [21,22]. Thus, *db/db* mice, which lack the leptin receptor, are appropriate for us to rule out the possible involvement of the interactions between these two adipokines. In the first place, we administered recombinant adiponectin to *db/db* mice intraperitoneally. The administration of adiponectin caused significant suppression of SREBP1c mRNA at 4 h in the liver (*left panel*, Fig. 1). At 8 h, the mRNA expressions of ACC-1 and SCD-1, the genes involved in fatty acid synthesis and regulated by SREBP1c expression, were also reduced (*right panel*, Fig. 1). The suppression of these genes was not attributed to the changes in the plasma insulin or glucose concentrations, both of which remained unchanged during the entire time course as expected (data not shown).

Adiponectin suppressed the expression of SREBP1c and lipogenic enzymes in Fao cells

Next we stimulated Fao cells, a well differentiated hepatoma cell line [23] with adiponectin. Adiponectin suppressed the expressions of SREBP1c in a time-dependent manner (*upper left panel*,

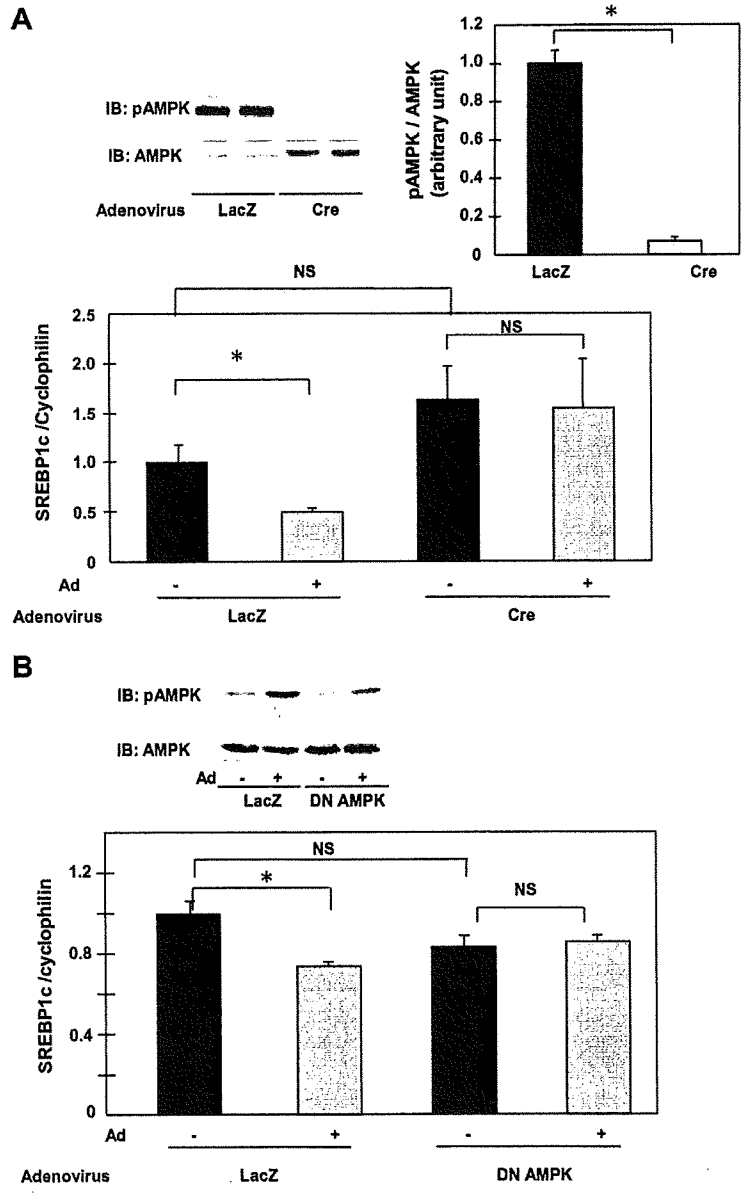

Fig. 4. Inhibition of AMPK phosphorylation abolishes SREBP1c suppression by adiponectin. (A, *upper panel*) Gene deletion of LKB1 was performed by adenovirus-mediated Cre expression in *LKB1*$^{lox/lox}$ mice. The panels show the representative blot and quantification of the phosphoAMPK/AMPK intensity of immunoblot analysis. Each bar represents the mean ± SE (subjects, $n = 5$; control, $n = 4$; $^{*}P < 0.05$). (A, *lower panel*) The mRNA expression of SREBP1c was measured 4 h after adiponectin treatment under LKB1 gene deletion. Each bar represents the mean ± SE ($n = 5$ except for the Cre-treated control mice, where the number of mice is 4, $^{*}P < 0.05$). (B, *upper panel*) The Fao cells were incubated with adiponectin under adenovirus-mediated overexpression of LacZ or DN-AMPK. The panels show the blot of the phosphoAMPK/AMPK. (B, *lower panel*) The mRNA expression of SREBP1c was measured in the Fao cells after 8 h incubation with adiponectin. Each bar represents the mean ± SE ($n = 3$, $^{*}P < 0.05$).

Fig. 2), and of other lipogenic genes such as ACC-1 and FAS at 8 h (*right panel*, Fig. 2). Next we conducted the reporter assay by using the plasmid harboring putative promoter region of SREBP1c (−2600-0 bp). In Fao cells, adiponectin significantly suppressed the SREBP1c promoter activity at 4 h (*lower left panel*, Fig. 2), consistent with the decrease in its mRNA expression. These *in vitro* assays strongly suggest that adiponectin directly suppresses the expressions of SREBP1c and its downstream enzymes in hepatocytes.

Adenovirus-mediated gene transfer of siAdipoR1 upregulated SREBP1c expression in the liver of db/db mice

Next we examined whether adiponectin suppressed SREBP1c by its functional receptors: AdipoR1 and AdipoR2. AdipoR1 has been reported to be ubiquitously expressed in various tissues, while AdipoR2 relatively abundant in the liver [13]. We constructed adenoviruses encoding siRNA for AdipoR1 and AdipoR2, and examined which receptor was responsible for the suppression of SREBP1c. Adenovirus-mediated gene transfer of siAdipoR1 and siAdipoR2 caused robust suppression of the respective receptor in the liver of *db/db* mice (*left panels*, Fig. 3A and B). With AdipoR1 suppressed, the expression of SREBP1c mRNA was significantly upregulated, and the SCD-1 mRNA expression also tended to be increased (*right panel*, Fig. 3A). There were no differences in the plasma glucose levels between the two groups (70.8 ± 7.0 vs. 72.8 ± 4.4 mg/dl, $n = 13$, $P = 0.81$). The plasma insulin concentration tended to be higher in the siAdipoR1-treated mice, though the difference did not reach a statistical significance (3221 ± 814.4 vs. 5378 ± 1041 pg/ml, $n = 13$, $P = 0.102$). On the other hand, the suppression of AdipoR2 did not alter SREBP1c expression in the liver, while the expressions of ACO and glucokinase (GCK) were significantly reduced (*right panel*, Fig. 3B). These results indicate that the suppression of SREBP1c by adiponectin is mediated by its functional receptor, and that AdipoR1, not AdipoR2, could account for the action. These are consistent with our recent report, showing that AdipoR1 knockout mice and AdipoR1/R2 double knockout mice showed elevated hepatic SREBP1c expression, while AdipoR2 knockout mice did not [24].

Adiponectin suppressed SREBP1c expression via activating AMPK in hepatocytes

Next, we investigated the mechanisms whereby adiponectin suppressed SREBP1c. It has been reported that adiponectin activates AMPK via AdipoR1 [12,24]. 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a pharmacological activator of AMPK, suppresses SREBP1c in hepatocytes [25], while the disruption of LKB1, one of the major upstream kinases of AMPK, caused a nearly complete loss of AMPK phosphorylation in the liver and led to significant elevation of the lipogenic genes [17]. These data prompted us to investigate whether adiponectin suppressed SREBP1c in hepatocytes via LKB1/AMPK pathway. LKB1 disruption in the liver was conducted by injecting an adenovirus expressing Cre recombinase to *LKB1*$^{lox/lox}$ mice, which led to robust inhibition of the basal AMPK phosphorylation (*upper panel*, Fig. 4A). LKB1 deletion led to elevated basal SREBP1c expression, and completely abolished the adiponectin-induced suppression of SREBP1c (*lower panel*, Fig. 4A). We also overexpressed the dominant negative mutant of AMPKα1 subunit (DN-AMPK) in Fao cells, and confirmed that the adiponectin-induced suppression of SREBP1c was abolished (Fig. 4B). These data suggest that adiponectin suppresses SREBP1c expression through the pathway composed of AdipoR1, LKB1 and AMPK, and also show that the AMPK activation by adiponecctin necessitates LKB1 as its upstream kinase.

What are the implications of SREBP1c suppression by adipnectin? Under physiological conditions, plasma glucose and insulin stimulates SREBP1c expression and fatty acid synthesis in the fed state, whereby excess energy is stored in the form of triglycerides in the liver. In our observation, plasma adiponectin concentration and the expressions of AdipoRs are elevated in the fasted state (unpublished data), suggesting that adiponectin action is physiologically more potent in the fasted state. Accordingly it is hypothesized that the suppression of SREBP1c by adiponectin could minimize excess energy storage in the liver in the fasted state, thereby allowing peripheral tissues to efficiently utilize the lipids as an energy source.

In contrast, under pathological conditions, the low expression of adiponectin and its receptors is thought to contribute to the pathogenesis of insulin resistance and the metabolic syndrome [8–11,26]. As shown in the current study, the attenuated action of adiponectin leads to upregulated SREBP1c expression, which is supposed to be one of the causal factors of fatty liver and insulin resistance. Interestingly, fatty acid synthesis is rather increased due to the compensatory hyperinsulinaemia, despite the downregulation of many insulin actions [5,27]. Our data suggest that the decreased adiponectin action could also, at least in part, contribute to the development of this paradoxical increase in SREBP1c expression and hepatic lipid accumulation in obesity-induced insulin resistance, although the precise elucidation awaits further experiments.

In summary, we here report for the first time that adiponectin suppresses SREBP1c expression in hepatocytes via AMPK activation through AdipoR1. The possible regulation of fatty acid synthesis by adiponectin, together with enhanced fatty acid oxidation, could be one of the mechanisms whereby adiponectin maintains insulin sensitivity.

Acknowledgments

We thank Dr. H. Shimano for the reporter plasmid used for the measurement of SREBP1c promoter activity, and F. Takahashi and H. Chiyonobu for their excellent technical assistance. This work was supported by a Grant-in-Aid for Creative Scientific Research from the Japan Society for the Promotion of Science (10NP0201) (to T.K.), a grant from Special Coordination Funds for Promoting Science and Technology, a grant from the Juvenile Diabetes Foundation International (1-2003-746) (to T.K.), a Grant-in-Aid for the Development of Innovative Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.K. and K.U.), Health Science Research grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare, a grant from Takeda Science Foundation (to K.U.).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 3, pp. 1803–1812, January 16, 2009

Rimonabant Ameliorates Insulin Resistance via both Adiponectin-dependent and Adiponectin-independent Pathways*

Received for publication, September 15, 2008, and in revised form, October 23, 2008 Published, JBC Papers in Press, November 13, 2008, DOI 10.1074/jbc.M807120200

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Rimonabant has been shown to not only decrease the food intake and body weight but also to increase serum adiponectin levels. This increase of the serum adiponectin levels has been hypothesized to be related to the rimonabant-induced amelioration of insulin resistance linked to obesity, although experimental evidence to support this hypothesis is lacking. To test this hypothesis experimentally, we generated adiponectin knock-out (*adipo*(−/−))ob/ob mice. After 21 days of 30 mg/kg rimonabant, the body weight and food intake decreased to similar degrees in the ob/ob and *adipo*(−/−)-ob/ob mice. Significant improvement of insulin resistance was observed in the ob/ob mice following rimonabant treatment, associated with significant up-regulation of the plasma adiponectin levels, in particular, of high molecular weight adiponectin. Amelioration of insulin resistance in the ob/ob mice was attributed to the decrease of glucose production and activation of AMP-activated protein kinase (AMPK) in the liver induced by rimonabant but not to increased glucose uptake by the skeletal muscle. Interestingly, the rimonabant-treated *adipo*(−/−)ob/ob mice also exhibited significant amelioration of insulin resistance, although the degree of improvement was significantly lower as compared with that in the ob/ob mice. The effects of rimonabant on the liver metabolism, namely decrease of glucose production and activation of AMPK, were also less pronounced in the *adipo*(−/−)-ob/ob mice. Thus, it was concluded that rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways.

The prevalence of obesity has increased dramatically in recent years (1, 2). It is commonly associated with type 2 diabetes, coronary artery disease, and hypertension, and the coexistence of these diseases in subjects has been termed the metabolic syndrome (3). There is a demand for effective and safe anti-obesity agents that can produce and maintain weight loss and improve the metabolic syndrome.

The newly discovered endocannabinoid system, consisting of the CB-1 (cannabinoid type-1) receptor and endogenous lipid-derived ligands, contributes to the physiological regulation of energy balance, food intake, and lipid and glucose metabolism, through both central orexigenic effects and peripheral metabolic effects (4–11). The endocannabinoid system is overactivated in genetic animal models of obesity (4, 6), and the selective CB-1 blocker, rimonabant, produces weight loss and ameliorates metabolic abnormalities in obese animals (12, 13). Patients with obesity and hyperglycemia associated with type 2 diabetes exhibit higher concentrations of endocannabinoids in the visceral fat and serum, respectively, than the corresponding controls (14). Rimonabant has been shown to produce substantial weight loss and reduction of waist circumference and also improve insulin resistance and the profile of several metabolic and cardiovascular risk factors in diabetic as well as nondiabetic obese patients (15–18).

Adiponectin is an adipokine that is specifically and abundantly expressed in the adipose tissue and released into the circulation, which directly sensitizes the body to insulin (19, 20). Administration of recombinant adiponectin to rodents increases the glucose uptake and fat oxidation in muscle, reduces hepatic glucose production, and improves whole body insulin sensitivity (21–23). Adiponectin-deficient (*adipo*(−/−)) mice exhibit insulin resistance and glucose intolerance (24, 25). Previous stud-

* This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (for the Translational Systems Biology and Medicine Initiative), Grant-in-Aid for Scientific Research in Priority Areas (A) 16209030 and (A) 18209033 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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