Table 1: Characteristics of study subjects.

	Total	Continuously lean	Previous obesity	Current obesity	P value
Age	59.3 ± 10.6	61.1 ± 9.4	60.4 ± 10.0	56.7 ± 11.6	<0.01
Sex (male/female)	2038/889	486/243	870/345	682/301	ns
BMI	24.0 ± 4.0	20.5 ± 2.1	22.6 ± 1.7	28.3 ± 3.2	<0.01
Max. BMI	27.7 ± 4.3	23.0 ± 1.7	27.5 ± 2.3	31.4 ± 4.1	<0.01
Disease duration (years)	10.4 ± 28.0	10.4 ± 8.4	12.2 ± 42.2	8.2 ± 7.8	<0.01
SBP (mmHg)	132 ± 18	131 ± 17	132 ± 18	133 ± 18	ns
DBP (mmHg)	75 ± 11	74 ± 11	75 ± 11	75 ± 10	ns
FPG (mmol/L)	9.9 ± 4.0	9.5 ± 4.0	9.9 ± 4.1	10.2 ± 3.9	ns
HbA1c (%)	9.3 ± 2.8	9.1 ± 2.7	9.5 ± 3.4	9.3 ± 2.0	<0.05
TC (mmol/L)	5.3 ± 1.2	5.3 ± 1.0	5.2 ± 1.0	5.5 ± 1.6	<0.01
HDL (mmol/L)	1.3 ± 0.5	1.5 ± 0.5	1.3 ± 0.4	1.2 ± 0.3	<0.01
TG (mmol/L)	1.6 ± 1.4	1.4 ± 0.2	1.5 ± 1.1	1.9 ± 1.6	<0.01
Cr (mmol/L)	66.3 ± 19.4	64.5 ± 16.8	66.3 ± 19.4	68.9 ± 20.3	<0.01
eGFR (mL/min/1.73 m^2)	81.8 ± 28.8	81.4 ± 20.5	83.1 ± 31.1	80.6 ± 31.0	ns
Retinopathy (none/simple/proliferative)	2111/505/311	558/116/55	824/225/166	729/164/90	<0.01

Continuous variables are expressed as mean ± SD. Differences in baseline characteristics among the obesity categories were analyzed by ANOVA and chi-squared test. The numbers of each stage of diabetic retinopathy are noted on the row of retinopathy. SBP: systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting blood glucose, TC: total cholesterol, HDL: HDL cholesterol, TG: triglyceride, ns: not significant.

Table 2: Logistic regression analysis with forced entry method for diabetic nephropathy.

	Odds ratio	95% confidence interval	P value
Age	1.011	1.002–1.019	<0.05
Disease duration	1.001	0.998–1.004	ns
Sex (male: 1, female: 0)	1.955	1.606–2.378	<0.001
Hypertension	1.232	1.023–1.484	<0.05
Dyslipidemia	1.306	1.096–1.556	<0.01
HbA1c	1.014	0.984–1.044	ns
Diabetic retinopathy	3.856	3.214–4.626	<0.001
Previous obesity: 1, other: 0	1.656	1.323–2.073	<0.001
Current obesity: 1, other: 0	2.480	1.959–3.141	<0.001

Adjusted $R^2 = 0.166$, $P < 0.001$. ns: not significant.

not the duration. So the hypothesis needs to be confirmed in a future large cohort study with more detailed information. In spite of these limitations, this was an analysis of a large population over 10 years. We therefore believe it includes important suggestions on the effect of obesity on diabetic nephropathy.

5. Conclusion

Our study indicated that obesity in the past, as well as present obesity, was a risk factor for diabetic nephropathy. We should consider the effect of earlier obesity on diabetic nephropathy even if it has been present before the diagnosis of diabetes.

Acknowledgments

The authors thank Ms. E. Inoue, and all the staff at Keio University Hospital for their assistance. They also thank all the staff at Saiseikai Central Hospital for their assistance.

References

[1] K. Kosaka and Y. Ogata, "Obesity and diabetes mellitus," *Saishin Igaku*, vol. 16, no. 10, pp. 2602–2608, 1961 (Japanese).

[2] H. Sone, Y. Yoshimura, H. Ito, Y. Ohashi, and N. Yamada, "Energy intake and obesity in Japanese patients with type 2 diabetes," *The Lancet*, vol. 363, no. 9404, pp. 248–249, 2004.

[3] J. Y. Park, K. Lee, C. H. Kim et al., "Past and current obesity in Koreans with non-insulin-dependent diabetes mellitus," *Diabetes Research and Clinical Practice*, vol. 35, no. 1, pp. 49–56, 1997.

[4] T. Nishimura, K. Nakajima, H. Kusuoka, A. Yamashina, and S. Nishimura, "Prognostic study of risk stratification among Japanese patients with ischemic heart disease using gated myocardial perfusion SPECT: J-ACCESS study," *European Journal of Nuclear Medicine and Molecular Imaging*, vol. 35, no. 2, pp. 319–328, 2008.

[5] M. Haffner, S. Lehto, T. Rönnemaa, K. Pyörälä, and M. Laakso, "Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction," *The New England Journal of Medicine*, vol. 339, no. 4, pp. 229–234, 1998.

[6] H. Sone, S. Tanaka, S. Iimuro et al., "Long-term lifestyle intervention lowers the incidence of stroke in Japanese patients with type 2 diabetes: a nationwide multicentre randomised controlled trial (the Japan Diabetes Complications Study)," *Diabetologia*, vol. 53, no. 3, pp. 419–428, 2010.

[7] UK Prospective Diabetes Study (UKPDS) Group, "Intensive blood-glucose control with sulphonylureas or insulin compared

with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)," *The Lancet*, vol. 352, no. 9131, pp. 837–853, 1998.

[8] R. R. Holman, S. K. Paul, M. A. Bethel, D. R. Matthews, and H. A. W. Neil, "10-year follow-up of intensive glucose control in type 2 diabetes," *The New England Journal of Medicine*, vol. 359, no. 15, pp. 1577–1589, 2008.

[9] M. Gall, P. Hougaard, K. Borch-Johnsen, and H. Parving, "Risk factors for development of incipient and overt diabetic nephropathy in patients with non-insulin dependent diabetes mellitus: prospective, observational study," *British Medical Journal*, vol. 314, no. 7083, pp. 783–788, 1997.

[10] M. Ravid, D. Brosh, D. Ravid-Safran, Z. Levy, and R. Rachmani, "Main risk factors for nephropathy in type 2 diabetes mellitus are plasma cholesterol levels, mean blood pressure, and hyperglycemia," *Archives of Internal Medicine*, vol. 158, no. 9, pp. 998–1004, 1998.

[11] R. Retnakaran, C. A. Cull, K. I. Thorne, A. I. Adler, and R. R. Holman, "Risk factors for renal dysfunction in type 2 diabetes: U.K. prospective diabetes study 74," *Diabetes*, vol. 55, no. 6, pp. 1823–1939, 2006.

[12] K. Ogawa, K. Ueda, H. Sasaki et al., "History of obesity as a risk factor for both carotid atherosclerosis and microangiopathy," *Diabetes Research and Clinical Practice*, vol. 66, supplement 1, pp. S165–S168, 2004.

[13] T. Kadowaki, Y. Miyake, R. Hagura et al., "On the pathogenesis of type II diabetes with special reference to diminished insulin response and obesity: a 5–12 year follow-up study of subjects with borderline glucose tolerance," *Tohoku Journal of Experimental Medicine*, vol. 141, pp. 141–146, 1983.

[14] Examination Committee of Criteria for "Obesity Disease" in Japan, Japan Society for the Study of Obesity, "New criteria for "obesity disease" in Japan," *Circulation*, vol. 66, no. 11, pp. 987–992, 2002.

[15] A. Kashiwagi, M. Kasuga, E. Araki et al., "International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values," *Journal of Diabetes Investigation*, vol. 3, no. 1, pp. 8–10, 2012.

[16] S. Matsuo, E. Imai, M. Horio et al., "Revised equations estimated GFR from serum creatinine," *American Journal of Kidney Diseases*, vol. 53, no. 6, pp. 982–992, 2009.

[17] D. Davis, T. Kern, and L. I. Rand, "Diabetic retinopathy," in *International Textbook of Diabetes Mellitus*, K. G. M. M. Alberti, P. Zimmet, and R. A. DeFronzo, Eds., pp. 1413–1446, John Wiley & Sons, Chichester, UK, 2nd edition, 1997.

[18] T. Ninomiya, V. Perkovic, B. E. de Galan et al., "Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes," *Journal of the American Society of Nephrology*, vol. 20, no. 8, pp. 1813–1821, 2009.

[19] S. Meguro, T. Shigihara, Y. Kabeya, M. Tomita, and Y. Atsumi, "Increased risk of renal deterioration associated with low e-GFR in type 2 diabetes mellitus only in albuminuric subjects," *Internal Medicine*, vol. 48, no. 9, pp. 657–663, 2009.

[20] R. A. Hoefield, P. A. Kalra, P. G. Baker et al., "The use of eGFR and ACR to predict decline in renal function in people with diabetes," *Nephrology Dialysis Transplantation*, vol. 26, no. 3, pp. 887–892, 2011.

[21] H. Yokoyama, S. Kanno, S. Takahashi et al., "Risks for glomerular filtration rate decline in association with progression of albuminuria in type 2 diabetes," *Nephrology Dialysis Transplantation*, vol. 26, no. 9, pp. 2924–2930, 2011.

[22] S. Meguro, M. Tomita, Y. Kabeya et al., "Factors associated with the decline of kidney function differ among eGFR strata in subjects with type 2 diabetes mellitus," *International Journal of Endocrinology*, vol. 2012, Article ID 687867, 6 pages, 2012.

[23] W. R. Ross and J. B. McGill, "Epidemiology of obesity and chronic kidney disease," *Advances in Chronic Kidney Disease*, vol. 13, no. 4, pp. 325–335, 2006.

[24] A. Vivante, E. Golan, D. Tzur et al., "Body mass index in 1.2 million adolescents and risk for end-stage renal disease," *Archives of Internal Medicine*, vol. 172, no. 21, pp. 1644–1650, 2012.

OPEN ACCESS Freely available online

PLOS ONE

C-Reactive Protein, High-Molecular-Weight Adiponectin and Development of Metabolic Syndrome in the Japanese General Population: A Longitudinal Cohort Study

Yoshifumi Saisho[1]*, Hiroshi Hirose[2], Rachel Roberts[3], Takayuki Abe[3], Hiroshi Kawabe[2], Hiroshi Itoh[1]

1 Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, **2** Health Center, Keio University School of Medicine, Tokyo, Japan, **3** Center for Clinical Research, Keio University School of Medicine, Tokyo, Japan

Abstract

Aims: To clarify predictive values of C-reactive protein (CRP) and high-molecular-weight (HMW) adiponectin for development of metabolic syndrome.

Research Design and Methods: We conducted a prospective cohort study of Japanese workers who had participated in an annual health checkup in 2007 and 2011. A total of 750 subjects (558 men and 192 women, age 46±8 years) who had not met the criteria of metabolic syndrome and whose CRP and HMW-adiponectin levels had been measured in 2007 were enrolled in this study. Associations between CRP, HMW-adiponectin and development of metabolic syndrome after 4 years were assessed by logistic regression analysis and their predictive values were compared by receiver operating characteristic analysis.

Results: Among 750 subjects, 61 (8.1%) developed metabolic syndrome defined by modified National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) criteria and 53 (7.1%) developed metabolic syndrome defined by Japan Society for the Study of Obesity (JASSO) in 2011. Although CRP and HMW-adiponectin were both significantly correlated with development of metabolic syndrome, multivariate logistic regression analysis revealed that HMW-adiponectin but not CRP was associated with metabolic syndrome independently of BMI or waist circumference. Adding these biomarkers to BMI or waist circumference did not improve the predictive value for metabolic syndrome.

Conclusion: Our findings indicate that the traditional markers of adiposity such as BMI or waist circumference remain superior markers for predicting metabolic syndrome compared to CRP, HMW-adiponectin, or the combination of both among the Japanese population.

Citation: Saisho Y, Hirose H, Roberts R, Abe T, Kawabe H, et al. (2013) C-Reactive Protein, High-Molecular-Weight Adiponectin and Development of Metabolic Syndrome in the Japanese General Population: A Longitudinal Cohort Study. PLoS ONE 8(9): e73430. doi:10.1371/journal.pone.0073430

Editor: Hideharu Abe, University of Tokushima, Japan

Received March 8, 2013; **Accepted** July 22, 2013; **Published** September 12, 2013

Funding: This study was supported in part by a Grant-in Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to H.H.), and by research grants from Keio University, Tokyo (to H.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Fujirebio Co., Tokyo and Keio University, Tokyo have a partial patent concerning HMW-adiponectin measurement kit ("Methods for diagnosis or monitoring of impaired glucose tolerance", WO2003/016906, JP-3624216 (2004) and JP-4214202 (2008)). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: ysaisho@z5.keio.jp

Introduction

Metabolic syndrome is now widely appreciated as a cluster of metabolic abnormalities such as visceral obesity, hypertension, hyperglycemia and dyslipidemia [1]. To date, incidence of metabolic syndrome is continuously increasing worldwide. Since subjects with metabolic syndrome are at risk for development of type 2 diabetes, cardiovascular disease (CVD) and cancer [1,2], prevention of metabolic syndrome is an urgent issue.

Higher levels of C-reactive protein (CRP) and lower levels of high-molecular-weight (HMW) adiponectin have been both reported to correlate with obesity and metabolic syndrome [3,4,5,6]. These biomarkers have been also reported to correlate with type 2 diabetes and CVD [5,7,8,9,10,11]. Therefore, these biomarkers appear to be useful to detect subjects at high risk of metabolic syndrome, type 2 diabetes and CVD.

Although associations between CRP or HMW-adiponectin and metabolic syndrome have been well established, there have been few studies to examine the usefulness of combination of both biomarkers for prediction of metabolic syndrome. Especially, in clinical settings it is essential to compare the predictive utility of these biomarkers for metabolic syndrome with that of the traditional markers. Therefore, in this longitudinal cohort study we sought to address the following questions: 1) Is there any additive effect of the combination of CRP and HMW-adiponectin for prediction of metabolic syndrome compared with each of them

alone? 2) Are predictive values of the traditional markers such as BMI or waist circumference for metabolic syndrome improved by adding these biomarkers?

Methods

Ethics Statement

The present study was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each subject after a full explanation of the purpose, nature and risk of all procedures used. The protocol was approved by the ethical review committees of the Health Center and the Faculty of Medicine, Keio University School of Medicine, Tokyo, Japan.

Subjects

We conducted a prospective cohort study of 1,552 Japanese teachers and workers at Keio University (1,067 men and 485 women) who had participated in an annual health checkup in 2007. More than 95% of the workers and teachers at Keio University participated in this annual health checkup. In this study, we primarily enrolled subjects aged 40 years and older because the incidence of metabolic syndrome is low in the subjects younger than 40 years old [12]. Then we randomly enrolled subjects younger than 40 years old. As a result, we measured CRP and HMW-adiponectin in 1,250 subjects out of 1,552 subjects (81%). Among them, 42 subjects were excluded because of their CRP>0.5 mg/L. Of those (1,208 subjects), 884 subjects (73%, 676 men and 208 women) had been followed up in an annual health checkup in 2011. Finally, a total of 750 subjects (558 men and 192 women, age 46±8 years) who had not met the criteria of metabolic syndrome defined by National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) and Japan Society for the Study of Obesity (JASSO) in 2007 were enrolled in this study (Table 1). The subjects with whom we were not able to follow up in 2011 (N = 324) were older (49±14 vs. 47±8 years, $p<0.05$) and predominantly female (32.4% vs. 23.5%, $p<0.05$) than the study participants (N = 884), although the difference was clinically small, presumably reflecting retirement or resignation from work. Information regarding smoking, alcohol intake, physical activity and medication was obtained from questionnaires to the subjects.

Measurement

Systolic and diastolic blood pressure was measured in the sitting position after resting for at least 3 min using an automatic electronic sphygmomanometer (BP-103i II; Nippon Colin, Komaki, Japan) with cuff size of 14.5 cm in width and 52.0 cm in length. Blood samples were collected in the morning after an overnight fast.

Plasma glucose and serum lipids were assayed by routine automated laboratory methods as previously described [13]. Serum insulin concentration was measured by an enzyme immunoassay, using a commercially available kit (Tosoh, Tokyo, Japan), with intra- and interassay coefficients of 2.9 to 4.6% and 4.5 to 7.0%, respectively. The insulin resistance index was assessed by a homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated as fasting serum insulin (mU/L) × fasting plasma glucose (mmol/L)/22.5 [14].

HMW-adiponectin was measured using a commercially available kit (HMW adiponectin ELISA Kit, Fujirebio, Tokyo, Japan) as previously reported [13]. This ELISA system does not need a denaturing step, and the monoclonal antibody (IH7) is reported to react specifically with the HMW form of adiponectin [15]. The dilution curve was parallel to the standard curve. Intra- and interassay coefficients were 2.4 to 3.0% and 4.2 to 5.1%, respectively. We have previously reported that HMW-adiponectin and HMW-adioponectin to total adiponectin ratio were more sensitively associated with metabolic syndrome than total adiponectin alone [16,17,18]. Thus we measured HMW-adiponectin rather than total adiponectin in this study. Serum CRP levels were measured by nephelometry, a latex particle-enhanced immunoassay (N Latex CRP II, Dade Behring, Tokyo, Japan) with both intra- and interassay coefficients of <5.0%. For the analysis of CRP values under the assay detection limit of 0.04 mg/L, an approximate value of 0.02 mg/L was used.

Definition of Metabolic Syndrome

Metabolic syndrome (MetS) was defined according to the revised NCEP ATP III criteria [1] as 3 or more of 5 components in which the cut-off point of waist circumference was modified for Japanese as ≥90 cm in men and ≥80 cm in women according to the recommendation by the International Diabetes Federation (IDF) [19], the cut-off points of the other components were systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg for blood pressure, ≥150 mg/dL for triglycerides, <40 mg/dL in men and <50 mg/dL in women for HDL-cholesterol, and ≥100 mg/dL for fasting plasma glucose. Subjects receiving antihypertensive, lipid-lowering agent or hypoglycemic medication were considered to have the respective component. Japanese metabolic syndrome (JMetS) defined by the Examination Committee for Criteria of Metabolic Syndrome [20] was also examined. The criteria of JMetS is waist circumference ≥85 cm in men and ≥90 cm in women plus 2 or more of the following 3 components; systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg, triglycerides ≥150 mg/dL and/or HDL cholesterol <40 mg/dL, and fasting plasma glucose ≥110 mg/dL.

Statistical Analysis

Comparisons between the two groups were performed with Student's t-tests or Fisher's exact tests and odds ratios were determined by logistic regression analysis using the Statistical Package for the Social Sciences (version 19.0; SPSS, Chicago, IL, USA). Receiver operating characteristic (ROC) curves for MetS and JMetS were plotted separately and the area under the curve (AUC) (also referred to as C-statistic in the case of a binary outcome) of ROC curves was calculated. ROC analysis was performed using Proc Logistic in SAS 9.2 (SAS Institute, Cary, NC), which estimates AUC using the trapezoidal method of integration of the sensitivity curve. All normally distributed data are expressed as mean ± S.D., while non-normal data are expressed as median (interquartile range) and the logarithms of the non-normal data were used for the analyses. Values of $p<0.05$ were considered statistically significant.

Results

Baseline Characteristics of Subjects According to Development of Metabolic Syndrome

During 4 years, 61 subjects (8.1%, 51 men and 10 women) developed MetS and 53 (7.1%, 50 men and 3 women) developed JMetS, respectively. Comparisons of baseline characteristics according to the development of metabolic syndrome are shown in Table 1. CRP and C/A ratio were significantly higher, and HMW-adiponectin was significantly lower in subjects who developed metabolic syndrome compared with those who did not (all $p<0.001$). HMW-adiponectin and CRP are significantly

Table 1. Characteristics of subjects according to development of metabolic syndrome after 4 years.

	Total	MetS		JMetS	
		(−)	(+)	(−)	(+)
N	750	689	61	697	53
Male (%)	74.4	73.6	83.6	72.9	94.3†
Age (years)	46±8	46±8	49±8**	46±8	50±7†
Height (m)	1.68±0.08	1.68±0.08	1.69±0.08	1.68±0.08	1.69±0.06
Weight (kg)	63.4±15.5	62.7±10.5	71.2±10.6†	62.7±10.6	71.4±8.8†
BMI (kg/m^2)	22.4±2.9	22.2±2.7	24.9±3.0†	22.2±2.8	24.9±2.8†
Waist circumference (cm)	80.1±8.2	79.5±8.0	86.7±7.7†	79.6±8.0	87.5±6.6†
Systolic blood pressure (mmHg)	120±16	119±15	132±16†	119±15	132±14†
Diastolic blood pressure (mmHg)	75±11	75±11	84±11†	75±11	84±8†
Heart rate (bpm)	76±12	75±12	79±11*	75±12	79±11*
Current smoking (%)	8.9	9.1	6.6	8.8	11.3
Alcohol intake (≥20 g/day) (%)	21.3	20.6	29.5	19.8	41.5**
No exercise (<150 min/week) (%)	61.9	62.3	57.4	62.4	54.7
Antihypertensives (%)	6.4	4.8	24.6†	4.6	30.2†
Lipid-lowering agents (%)	1.7	1.2	8.2**	1.9	0.0
Oral hypoglycemic agents (%)	0.7	0.7	0.0	0.4	3.8*
AST (IU/L)	20 (17–24)	20 (17–24)	20 (17–25)	20 (17–23)	24 (19–28)**
ALT (IU/L)	20 (14–26)	19 (14–26)	24 (17–36)*	19 (14–26)	26 (20–36)**
γ-GTP (IU/L)	26 (17–42)	25 (17–40)	37 (26–71)*	24 (17–40)	47 (32–80)**
ALP (IU/L)	198±59	198±58	206±62	198±59	201±58
Glucose (mg/dL)	89 (85–94)	89 (85–94)	94 (89–97)*	89 (85–94)	94 (91–98)**
Total cholesterol (mg/dL)	206±30	205±29	216±35*	206±30	207±30
LDL-cholesterol (mg/dL)	121±28	120±27	135±35**	121±27	123±32
HDL-cholesterol (mg/dL)	64±16	64±16	56±12†	64±16	55±11†
Triglyceride (mg/dL)	79 (53–113)	76 (52–110)	114 (84–148)†	75 (52–109)	123 (97–149)†
Uric acid (mg/dL)	5.6±1.4	5.6±1.4	6.3±1.1†	5.6±1.3	6.6±1.2†
Creatinine (mg/dL)	0.78±0.14	0.78±0.14	0.81±0.16	0.78±0.14	0.84±0.13**
Insulin (mU/L)	4.1 (3.0–5.8)	4.1 (3.0–5.7)	5.2 (3.9–7.1)	4.1 (3–5.6)	5.8 (3.9–7.4)*
HOMA-IR	0.91 (0.67–1.31)	0.89 (0.66–1.27)	1.18 (0.86–1.68)*	0.89 (0.66–1.26)	1.40 (0.94–1.75)**
CRP (mg/L)	0.28 (0.13–0.62)	0.26 (0.12–0.60)	0.54 (0.23–0.78)†	0.26 (0.12–0.6)	0.54 (0.33–0.74)†
HMW-adiponectin (μg/mL)	4.0 (2.5–6.3)	4.2 (2.6–6.5)	2.7 (1.8–3.9)†	4.3 (2.6–6.5)	2.3 (1.6–3.3)†
C/A ratio	0.08 (0.03–0.19)	0.07 (0.02–0.18)	0.20 (0.08–0.41)†	0.07 (0.02–0.18)	0.24 (0.10–0.41)†

Values are expressed as mean ± SD for variables with normal distribution and median (interquartile range) for those with non-normal distribution. MetS; metabolic syndrome, JMetS; Japanese metabolic syndrome.
*P<0.05,
**P<0.01,
†P<0.001 vs. MetS(−) or JMetS(−).
doi:10.1371/journal.pone.0073430.t001

correlated with BMI and waist circumference (Table 2), whereas the correlation was stronger in HMW-adiponectin compared with CRP.

Association between CRP, HMW-adiponectin and Development of Metabolic Syndrome

In univariate logistic regression analysis, both CRP and HMW-adiponectin were significantly associated with development of metabolic syndrome (odds ratio: 1.54 and 0.45 for MetS and 1.65 and 0.33 for JMetS, respectively, Table 3). However, when the model was adjusted for age, sex and traditional markers of adiposity such as BMI or waist circumference, HMW-adiponectin, but not CRP, was significantly associated with development of metabolic syndrome (Table 3). Adding the other variables which relate to development of metabolic syndrome to the model did not change the results (Models 3 and 4, Table S1).

Predictive Values of CRP and HMW-adiponectin for Metabolic Syndrome

Finally we evaluated additive effects of CRP and HMW-adiponectin on BMI and waist circumference for prediction of metabolic syndrome (Table 4). ROC analysis revealed that there is no significant change in the AUC of the combination of CRP and HMW-adiponectin compared with that of each of them alone. The AUC of BMI or waist circumference itself was greater than that of CRP, HMW-adiponectin or the combination of both.

Table 2. Correlations among HMW-adiponectin, CRP, BMI and waist circumference.

	BMI	Waist circumference	HMW-adiponectin	CRP
BMI	1	0.853**	−0.413**	0.194**
Waist circumference	0.853**	1	−0.409**	0.221**
HMW-adiponectin	−0.413**	−0.409**	1	−0.117*
CRP	0.194**	0.221**	−0.117*	1

*P = 0.001,
**P<0.001.
doi:10.1371/journal.pone.0073430.t002

Comparison between predictive ability of the models using CRP and HMW-adiponectin after adjusting for BMI or waist circumference, and the models using BMI or waist circumference alone, was done using the ROCCONTRAST statement in SAS. All but one of these comparisons showed no significant difference, but the only significant difference was not meaningful, as the actual difference in AUC was less than 1%. Thus the AUC of BMI or waist circumference was not significantly improved by adding CRP, HMW-adiponectin or both. These findings did not change when the subjects were stratified by sex (Tables S2 and S3).

Discussion

In this study, we report that 1) Combination of CRP and HMW-adiponectin did not improve predictive value for metabolic syndrome compared with each of them alone, 2) Adding these biomarkers to BMI or waist circumference failed to improve predictive value for metabolic syndrome among the Japanese population.

CRP and HMW-adiponectin are both well-known markers for metabolic syndrome, type 2 diabetes and CVD in various ethnics including Japanese [3,4,5,6,7,8,9,10,11]. In this study, we also showed that higher CRP and lower HMW-adiponectin were associated with future development of metabolic syndrome defined by either modified NCEP criteria or JASSO criteria. However, the association between metabolic syndrome and CRP, but not HMW-adiponectin, was markedly attenuated after adjustment for age, sex and BMI or waist circumference. It has been reported that CRP is more closely correlated with obesity than metabolic syndrome [21,22,23]. Our findings also suggested that the association between CRP and metabolic syndrome are largely explained by obesity.

On the other hand, the association between HMW-adiponectin and metabolic syndrome was significant independently of BMI or waist circumference. Adiponectin is an adipokine secreted from adipose tissue and negatively correlated with visceral obesity and presence of CVD [5,6,8,9,10,11]. In animal studies adiponectin has been shown to ameliorate metabolic parameters and suppress progression of atherosclerosis [24]. Specifically, we have shown, as well as others in the field that HMW-adiponectin is a more sensitive marker for metabolic syndrome [16,17,18,25] and type 2 diabetes [26,27] than total adiponectin. However, the usefulness of HMW-adiponectin for prediction of metabolic syndrome compared with the traditional markers remains uncertain. In this study, the predictive value of HMW-adiponectin for metabolic syndrome by ROC analysis was not significantly greater than that of BMI or waist circumference and adding HMW-adiponectin to BMI or waist circumference did not improve predictive ability, suggesting the lack of utility of HMW-adiponectin to predict future development of metabolic syndrome.

In this study, we further investigated the utility of combination of CRP and HMW-adiponectin for predicting metabolic syndrome. Tabara et al. have reported the synergistic effect of CRP and HMW-adiponectin for prediction of metabolic syndrome in a general population [28]. On the other hand, we and others have previously reported that C/A ratio did not improve the predictive ability for metabolic syndrome compared with each of them alone [13,29]. Recently, Ong et al. have reported that CRP and total

Table 3. Odds ratios (95% CI) according to univariate and multivariate logistic regression analyses of HMW-adiponectin and CRP for development of metabolic syndrome.

	MetS			JMetS		
	Univariate	Multivariate		Univariate	Multivariate	
Variable		Model 1	Model 2		Model 1	Model 2
Age (years)	1.05 (1.01–1.09)**	1.04 (1.00–1.08)*	1.03 (0.99–1.07)	1.09 (1.04–1.13)†	1.07 (1.03–1.12)**	1.07 (1.02–1.11)**
Sex (male = 1, female = 0)	1.83 (0.91–3.68)	0.55 (0.24–1.28)	0.60 (0.26–1.36)	6.20 (1.91–20.11)**	0.56 (0.16–1.98)	0.53 (0.15–1.88)
Ln{CRP (mg/L)}	1.54 (1.22–1.96)†	1.21 (0.92–1.59)	1.21 (0.92–1.59)	1.65 (1.28–2.13)†	1.29 (0.95–1.73)	1.26 (0.93–1.71)
Ln{HMW-adiponectin (μg/mL)}	0.45 (0.31–0.64)†	0.60 (0.38–0.96)*	0.56 (0.36–0.88)*	0.33 (0.22–0.49)†	0.50 (0.30–0.81)**	0.48 (0.30–0.78)**
BMI	1.37 (1.25–1.51)†	1.31 (1.18–1.46)†	–	1.36 (1.23–1.50)†	1.25 (1.12–1.39)†	–
Waist circumference (cm)	1.11 (1.07–1.15)†	–	1.09 (1.05–1.13)†	1.13 (1.09–1.17)†	–	1.09 (1.05–1.14)†

CI; confidence interval.
*P<0.05,
**P<0.01,
†P<0.001.
doi:10.1371/journal.pone.0073430.t003

Table 4. Comparison of predictive values of biomarkers for metabolic syndrome.

Variables	AUC of ROC curve (95% CI)	
	MetS	JMetS
CRP (mg/L)	0.646 (0.581–0.711)	0.683 (0.626–0.741)
HMW-adiponectin (ADPN) (μg/mL)	0.674 (0.610–0.738)	0.735 (0.671–0.799)
C/A ratio	0.698 (0.637–0.759)	0.753 (0.699–0.807)
ADPN+CRP	0.690 (0.627–0.752)	0.737 (0.673–0.800)
BMI (kg/m^2)	0.763 (0.711–0.816)	0.758 (0.705–0.811)
Waist circumference (WC) (cm)	0.794 (0.751–0.836)	0.754 (0.703–0.806)
BMI+WC	0.795 (0.751–0.840)	0.763 (0.712–0.814)
BMI+CRP	0.764 (0.712–0.817)	0.761 (0.708–0.813)
BMI+ADPN	0.795 (0.742–0.849)	0.769 (0.717–0.822)
BMI+C/A ratio	0.770 (0.719–0.822)	0.762 (0.710–0.814)
BMI+ADPN+CRP	0.795 (0.742–0.849)	0.772 (0.719–0.824)
WC+CRP	0.754 (0.702–0.806)	0.797 (0.755–0.839)
WC+ADPN	0.768 (0.713–0.822)	0.810 (0.761–0.859)
WC+C/A ratio	0.758 (0.707–0.810)	0.800 (0.759–0.842)
WC+ADPN+CRP	0.768 (0.714–0.823)	0.810 (0.761–0.859)

AUC; area under the curve, ROC; receiver operating characteristics, CI; confidence interval, C/A ratio; CRP to HMW-adiponectin ratio. Lines with variables written with "+" signs indicate that the ROC given is a measure of how well the combination of variables listed explain MetS or JMetS, where values closer to 1 indicate better explanation.

doi:10.1371/journal.pone.0073430.t004

adiponectin levels independently predict the deterioration of glycemia [30]. However, to our knowledge, there has been no study to investigate the utility of combination of both markers for predicting future development of metabolic syndrome in a longitudinal cohort. In the present study, we demonstrated that there is little additive effect of combination of CRP and HMW-adiponectin or C/A ratio for predicting metabolic syndrome compared with each of them alone.

Finally, we examined the additive effect of these biomarkers on the traditional markers for prediction of metabolic syndrome. As a result, ROC analysis revealed that adding CRP and HMW-adiponectin to the traditional markers did not improve the predictive ability for metabolic syndrome. Recently, it has been reported that inflammatory biomarkers including CRP failed to predict type 2 diabetes after adjustment for the traditional markers such as age, sex, BMI and waist circumference [31,32], whereas Ong et al., have reported that adding CRP and adiponectin to the traditional markers improved predictive ability for deterioration in glycemia, especially in women [30]. Further studies are needed to clarify whether the combination of CRP and HMW-adiponectin improve predictive ability for development of metabolic syndrome and type 2 diabetes.

There are limitations in this study. The relatively small number of subjects who developed metabolic syndrome in this study might reduce the ability to detect a statistical difference among the parameters. However, the high follow-up rate of this study (73%) suggested that the effect of selection bias was low. Second, the proportion of women in this study was relatively small and there were only 10 and 3 women who developed MetS and JMets respectively. Thus our findings in women should be confirmed in future studies with a larger sample size, although we found consistent results when we conducted subanalyses of men and women separately. Third, since the original criteria defined by JASSO is used for the diagnosis of metabolic syndrome in Japan, our findings may not apply to other countries or ethnicities which use different criteria of metabolic syndrome. However, we confirmed the findings by use of two different definitions of metabolic syndrome in this study. As we focused to the development of metabolic syndrome in this study, we were not able to exclude the possibility that CRP and HMW-adiponectin may be useful to predict each component of metabolic syndrome such as impaired glucose tolerance and dyslipidemia. Finally, since our study population was limited to healthy middle-aged Japanese (i.e., teachers and workers at University), the results of this study may not be applied to other population such as children and adolescents, and particularly elderly in which the higher adiponectin levels have been shown to associate with higher risk of CVD and mortality [33,34,35,36].

In conclusion, in this study we reported that CRP, HMW-adiponectin or the combination of both did not improve the predictive value of BMI and waist circumference for metabolic syndrome. Our findings indicate that the traditional markers of adiposity such as BMI or waist circumference are still superior markers for predicting metabolic syndrome among the Japanese population.

Supporting Information

Table S1 Odds ratios (95% CI) according to multivariate logistic regression analyses of HMW-adiponectin and CRP for development of metabolic syndrome.
(DOC)

Table S2 Comparison of predictive values of biomarkers for metabolic syndrome in men.
(DOC)

Table S3 Comparison of predictive values of biomarkers for metabolic syndrome in women.
(DOC)

Author Contributions

Conceived and designed the experiments: YS HH HK HI. Performed the experiments: YS HH. Analyzed the data: YS HH TA RR. Contributed reagents/materials/analysis tools: YS HH TA RR. Wrote the paper: YS. Interpretation of the data: YS HH RR TA HK HI. Reviewed/edited the manuscript: YS HH RR TA HK HI. Final approval of the version to be published: YS HH RR TA HK HI.

References

1. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, et al. (2009) Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 120: 1640–1645.
2. Esposito K, Chiodini P, Colao A, Lenzi A, Giugliano D (2012) Metabolic syndrome and risk of cancer: a systematic review and meta-analysis. Diabetes Care 35: 2402–2411.
3. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB (1999) Elevated C-reactive protein levels in overweight and obese adults. JAMA 282: 2131–2135.
4. Ford ES (2003) The metabolic syndrome and C-reactive protein, fibrinogen, and leukocyte count: findings from the Third National Health and Nutrition Examination Survey. Atherosclerosis 168: 351–358.

5. Yatagai T, Nagasaka S, Taniguchi A, Fukushima M, Nakamura T, et al. (2003) Hypoadiponectinemia is associated with visceral fat accumulation and insulin resistance in Japanese men with type 2 diabetes mellitus. Metabolism 52: 1274–1278.
6. Matsushita K, Yatsuya H, Tamakoshi K, Wada K, Otsuka R, et al. (2006) Comparison of circulating adiponectin and proinflammatory markers regarding their association with metabolic syndrome in Japanese men. Arterioscler Thromb Vasc Biol 26: 871–876.
7. Corrado E, Rizzo M, Coppola G, Fattouch K, Novo G, et al. (2010) An update on the role of markers of inflammation in atherosclerosis. J Atheroscler Thromb 17: 1–11.
8. Lindsay RS, Funahashi T, Hanson RL, Matsuzawa Y, Tanaka S, et al. (2002) Adiponectin and development of type 2 diabetes in the Pima Indian population. Lancet 360: 57–58.
9. Daimon M, Oizumi T, Saitoh T, Kameda W, Hirata A, et al. (2003) Decreased serum levels of adiponectin are a risk factor for the progression to type 2 diabetes in the Japanese Population: the Funagata study. Diabetes Care 26: 2015–2020.
10. Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, et al. (2004) Plasma adiponectin levels and risk of myocardial infarction in men. JAMA 291: 1730–1737.
11. Inoue T, Kotooka N, Morooka T, Komoda H, Uchida T, et al. (2007) High molecular weight adiponectin as a predictor of long-term clinical outcome in patients with coronary artery disease. Am J Cardiol 100: 569–574.
12. Ministry of Health, Labor and Welfare (Japan) (2007) National Health and Nutrition Survey.
13. Saisho Y, Hirose H, Seino Y, Saito I, Itoh H (2010) Usefulness of C-Reactive Protein to High-Molecular-Weight Adiponectin Ratio to Predict Insulin Resistance and Metabolic Syndrome in Japanese Men. J Atheroscler Thromb 17: 944–952.
14. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, et al. (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28: 412–419.
15. Nakano Y, Tajima S, Yoshimi A, Akiyama H, Tsushima M, et al. (2006) A novel enzyme-linked immunosorbent assay specific for high-molecular-weight adiponectin. J Lipid Res 47: 1572–1582.
16. Seino Y, Hirose H, Saito I, Itoh H (2007) High molecular weight multimer form of adiponectin as a useful marker to evaluate insulin resistance and metabolic syndrome in Japanese men. Metabolism 56: 1493–1499.
17. Seino Y, Hirose H, Saito I, Itoh H (2009) High-molecular-weight adiponectin is a predictor of progression to metabolic syndrome: a population-based 6-year follow-up study in Japanese men. Metabolism 58: 355–360.
18. Hirose H, Yamamoto Y, Seino-Yoshihara Y, Kawabe H, Saito I (2010) Serum high-molecular-weight adiponectin as a marker for the evaluation and care of subjects with metabolic syndrome and related disorders. J Atheroscler Thromb 17: 1201–1211.
19. Alberti KG, Zimmet P, Shaw J (2006) Metabolic syndrome–a new world-wide definition. A Consensus Statement from the International Diabetes Federation. Diabet Med 23: 469–480.
20. The Examination Committee for Criteria of Metabolic Syndrome (2005) Definition and criteria of metabolic syndrome. J Jpn Soc Intern Med 94: 794–809 (in Japanese).
21. Kahn SE, Zinman B, Haffner SM, O'Neill MC, Kravitz BG, et al. (2006) Obesity is a major determinant of the association of C-reactive protein levels and the metabolic syndrome in type 2 diabetes. Diabetes 55: 2357–2364.
22. Timpson NJ, Lawlor DA, Harbord RM, Gaunt TR, Day IN, et al. (2005) C-reactive protein and its role in metabolic syndrome: mendelian randomisation study. Lancet 366: 1954–1959.
23. Aronson D, Bartha P, Zinder O, Kerner A, Markiewicz W, et al. (2004) Obesity is the major determinant of elevated C-reactive protein in subjects with the metabolic syndrome. Int J Obes Relat Metab Disord 28: 674–679.
24. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, et al. (2003) Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. J Biol Chem 278: 2461–2468.
25. Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, et al. (2006) Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. Diabetes Care 29: 1357–1362.
26. Aso Y, Yamamoto R, Wakabayashi S, Uchida T, Takayanagi K, et al. (2006) Comparison of serum high-molecular weight (HMW) adiponectin with total adiponectin concentrations in type 2 diabetic patients with coronary artery disease using a novel enzyme-linked immunosorbent assay to detect HMW adiponectin. Diabetes 55: 1954–1960.
27. Basu R, Pajvani UB, Rizza RA, Scherer PE (2007) Selective downregulation of the high molecular weight form of adiponectin in hyperinsulinemia and in type 2 diabetes: differential regulation from nondiabetic subjects. Diabetes 56: 2174–2177.
28. Tabara Y, Osawa H, Kawamoto R, Tachibana-Iimori R, Yamamoto M, et al. (2008) Reduced high-molecular-weight adiponectin and elevated high-sensitivity C-reactive protein are synergistic risk factors for metabolic syndrome in a large-scale middle-aged to elderly population: the Shimanami Health Promoting Program Study. J Clin Endocrinol Metab 93: 715–722.
29. Devaraj S, Swarbrick MM, Singh U, Adams-Huet B, Havel PJ, et al. (2008) CRP and adiponectin and its oligomers in the metabolic syndrome: evaluation of new laboratory-based biomarkers. Am J Clin Pathol 129: 815–822.
30. Ong KL, Tso AW, Xu A, Law LS, Li M, et al. (2011) Evaluation of the combined use of adiponectin and C-reactive protein levels as biomarkers for predicting the deterioration in glycaemia after a median of 5.4 years. Diabetologia 54: 2552–2560.
31. Chao C, Song Y, Cook N, Tseng CH, Manson JE, et al. (2010) The lack of utility of circulating biomarkers of inflammation and endothelial dysfunction for type 2 diabetes risk prediction among postmenopausal women: the Women's Health Initiative Observational Study. Arch Intern Med 170: 1557–1565.
32. Dallmeier D, Larson MG, Wang N, Fontes JD, Benjamin EJ, et al. (2012) Addition of inflammatory biomarkers did not improve diabetes prediction in the community: the framingham heart study. J Am Heart Assoc 1: e000869.
33. Wannamethee SG, Whincup PH, Lennon L, Sattar N (2007) Circulating adiponectin levels and mortality in elderly men with and without cardiovascular disease and heart failure. Arch Intern Med 167: 1510–1517.
34. Kizer JR, Benkeser D, Arnold AM, Mukamal KJ, Ix JH, et al. (2012) Associations of total and high-molecular-weight adiponectin with all-cause and cardiovascular mortality in older persons: the Cardiovascular Health Study. Circulation 126: 2951–2961.
35. Kizer JR, Benkeser D, Arnold AM, Djousse L, Zieman SJ, et al. (2013) Total and high-molecular-weight adiponectin and risk of coronary heart disease and ischemic stroke in older adults. J Clin Endocrinol Metab 98: 255–263.
36. Saito I, Yamagishi K, Chei CL, Cui R, Ohira T, et al. (2013) Total and high molecular weight adiponectin levels and risk of cardiovascular disease in individuals with high blood glucose levels. Atherosclerosis Epub ahead of print.

Change in β-Cell Mass in Japanese Nondiabetic Obese Individuals

Kinsei Kou, Yoshifumi Saisho, Seiji Satoh, Taketo Yamada, and Hiroshi Itoh

Departments of Internal Medicine (K.K., Y.S., S.S., H.I.) and Pathology (T.Y.), Keio University School of Medicine, Tokyo 160–8582, Japan

Aim: The aim of this study was to clarify the change in β-cell mass in Japanese obese individuals.

Methods: We obtained the pancreas at autopsy from 39 lean and 33 obese Japanese nondiabetic individuals (aged 47 ± 13 vs 47 ± 12 y, $P = .83$, body mass index 20.4 ± 1.6 vs 28.5 ± 3.9 kg/m^2, $P < .01$). Pancreatic sections were stained for insulin, and β-cell area (%BCA) was measured as the fraction of the β-cell area to the total pancreas area. β-Cell mass was then calculated as the product of %BCA and estimated pancreas weight. β-Cell replication and apoptosis were assessed by double staining for insulin and Ki67 and insulin and single-stranded DNA, respectively. The frequencies of insulin-positive duct cells and scattered β-cells were assessed as the surrogate markers of β-cell neogenesis. The α-cell area (%ACA) was also measured, and the %ACA to %BCA ratio was determined.

Results: There was no increase in β-cell mass in obese individuals compared with lean individuals (0.6 ± 0.4 vs 0.7 ± 0.4 g, $P = .12$). β-Cell replication, β-cell neogenesis, and β-cell apoptosis were not significantly increased in the presence of obesity. There was no significant difference in %ACA to %BCA ratio between obese and lean individuals (0.91 ± 1.09 vs 0.75 ± 0.51, $P = .47$).

Conclusion: There was no increase in β-cell mass and no detectable change in β-cell turnover in Japanese obese individuals. (***J Clin Endocrinol Metab* 98: 3724–3730, 2013**)

Type 1 and type 2 diabetes are both characterized by a deficit of β-cell mass (BCM) (1–6). Preservation or recovery of BCM is therefore an important therapeutic strategy for both type 1 and type 2 diabetes. However, the physiological changes in BCM prior to the development of diabetes remain unclear.

Insulin secretion increases in the face of obesity to compensate insulin resistance (7). Although an increase in BCM has been reported in obese individuals (4, 8), there are no data available on Japanese individuals. There has been one report from Korea showing a positive correlation between fractional β-cell area and body mass index (BMI); however, the positive correlation was seen in only a small number of nondiabetic subjects (2). Recent studies have shown that insulin secretion in Japanese is lower than that in Caucasians (9–11), suggesting that the physiological response of BCM may also differ between Japanese and Caucasians. Therefore, in this study we sought to address the following questions: 1) is BCM increased in Japanese obese individuals; 2) is there any change in β-cell turnover in Japanese obese individuals; and 3) does α-cell area to β-cell area ratio change in Japanese obese individuals?

Materials and Methods

Subjects

Specimens of pancreas obtained at autopsy were obtained with the permission of the bereaved families. The Keio University School of Medicine Review Board approved this study.

Potential cases were first identified by retrospective analysis of the Keio University autopsy database. To be included, cases were required to have the following characteristics: 1) been aged

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received February 11, 2013. Accepted June 6, 2013.
First Published Online June 13, 2013

For editorial see page 3595

Abbreviations: %ACA, fractional α-cell area; %BCA, fractional β-cell area; BCM, β-cell mass; BMI, body mass index; HbA1c, glycated hemoglobin; ssDNA, single-stranded DNA.

Table 1. Characteristics of Subjects

	Total	Lean (BMI < 25 kg/m^2)	Obese (BMI ≥ 25 kg/m^2)	*P* Value
n	72	39	33	
Sex (male/female)	46/26	22/17	24/9	.23
Age, y	47 ± 12 (22–69)	47 ± 13 (22–69)	47 ± 12 (23–69)	.83
Height, cm	165 ± 9 (150–182)	164 ± 9 (150–182)	167 ± 9 (150–182)	.24
Weight, kg	66 ± 17 (44–132)	55 ± 6 (44–74)	80 ± 15 (58–132)	<.01
BMI, kg/m^2	24.1 ± 5.0 (16.1–44.3)	20.4 ± 1.6 (16.1–24.1)	28.5 ± 3.9 (25.2–44.3)	<.01

Data are expressed as mean ± SD (range).

20–69 years; 2) had a full autopsy within 24 hours of death; 3) medical information prior to death; 4) no history of diabetes, pancreatitis, or pancreatic surgery; 5) no use of glucocorticoids; and 6) pancreatic tissue stored that was of adequate size and quality. Cases were excluded if pancreatic tissue had undergone autolysis. As a result, we reviewed approximately 1000 autopsy cases between 1999 and 2011, and 72 cases were found to be eligible for this study. Most (n = 65) specimens were sampled from the body or tail portion of the pancreas and 7 specimens were sampled from the head of the pancreas. The characteristics of the cases are summarized in Table 1, with causes of death in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Cases were divided into 39 lean [body mass index (BMI) < 25 kg/m^2] and 33 obese (BMI ≥ 25 kg/m^2) cases according to the definition of obesity by the Japan Society for the Study of Obesity (12).

Pancreatic tissue processing

The pancreas was fixed in formaldehyde at autopsy and then embedded in paraffin for subsequent analysis. Then 5-μm sections were stained for light microscopy as follows: 1) with hematoxylin-eosin, 2) for insulin (peroxidase staining) with hematoxylin, 3) for glucagon with hematoxylin, 4) for insulin and Ki67 for assessment of β-cell replication, and 5) for insulin and single-stranded DNA (ssDNA) for assessment of β-cell apoptosis (13–15). For immunohistochemical staining, guinea pig polyclonal antibodies against porcine insulin and rabbit polyclonal antibodies against human glucagon were used (DAKO Japan, Kyoto, Japan). Furthermore, rabbit polyclonal antibodies against ssDNA (IBL, Takasaki, Japan) and murine monoclonal antibodies against human Ki67 (DAKO Japan) were used for the detection of apoptotic cells and proliferating cells, respectively. β-Cell apoptosis was also assessed by rabbit polyclonal antibodies against large fragment (17/19 kDa) of cleaved caspase-3 (Cell Signaling Technology, Boston, Massachusetts) and rabbit polyclonal antibodies against large fragment (89 kDa) of cleaved human poly(ADP-ribose) polymerase-1 (Cell Signaling Technology).

Morphometric analysis

To quantify fractional β-cell area (%BCA), the entire pancreatic section (219 ± 93 mm^2) was imaged at ×200 magnification (×20 objective) using a Mirax Scan and Mirax Viewer (Carl Zeiss MicroImaging GmbH, Goettingen, Germany). The ratio of β-cell area to total pancreas area was digitally measured using Image Pro Plus software (Media Cybernetics, Silver Spring, Maryland) as previously reported (8). Interlobular connective tissue, large blood vessels, and adipocytes were excluded from total pancreas area; thus, total pancreas area consisted to the greatest extent of pancreatic acinar tissue and pancreatic islets. Likewise, the ratio of α-cell area to total pancreas area (%ACA) was also digitally measured, and the ratio of %ACA to %BCA was determined in each case. All measurements were conducted by a single investigator (K.K.), and intraobserver coefficient of variance (computed in 5 cases studied on 5 occasions) was 7%. All measurements were conducted twice, and the mean of the 2 measurements was used. At the measurement the investigator was blinded to the BMI status of each specimen. In a preliminary analysis, the measurements of the %BCA independently assessed by 2 investigators (K.K. and Y.S.) were sufficiently comparable (n = 51, r = 0.9, $P < .01$, y (percentage) = 1.04x (percentage) + 0.1). Interobserver variance was about 12%.

To measure individual β-cell size, 6 islets per case were selected at random using a Mirax Viewer (Carl Zeiss MicroImaging). These islets were then examined to identify 6 representative β-cells in each. Selection criteria included a circular shape (similar dimensions in all directions) and the appearance to the observer that the cell had been sectioned through its maximum diameter. For the determination of the mean cell diameter, 6 distances between 2 adjacent β-cell nuclei (including one of the nuclei) were measured in each of the 6 islets (ie, total of 36 diameters in each case). The β-cell size was determined as mean β-cell diameter.

To conduct further morphometric analysis, scattered β-cells, insulin-positive duct cells, β-cell replication, and apoptosis were quantified in randomly selected areas of the pancreas (26.2 ± 13.5 mm^2) that contained more than 100 islets in each case, using a Mirax Viewer (Carl Zeiss MicroImaging). Scattered β-cells were defined as a cluster of three or fewer β-cells in acinar tissue, and the density of scattered β-cells was determined as the number of scattered β-cells/pancreas area (square millimeters). Insulin-positive duct cells were also counted and expressed as the number of insulin-positive duct cells/pancreas area (square millimeters). β-Cell replication and apoptosis were quantified, and the frequencies of β-cell replication and apoptosis were expressed as percentage of islets. A total of 7778 islets (108 ± 7 islets per section) were assessed for these analyses.

Pancreas parenchymal volume

Because pancreas weight was not available in most cases, to determine BCM, the pancreas parenchymal volume was estimated using equations based on population data described in detail elsewhere (16). Briefly, the pancreas parenchymal volume increases in childhood to reach a plateau at age 20 years. From age 20–60 years, pancreas parenchymal volume is stable and is described as a function of obesity. Thus, we estimated pancreas parenchymal volume of each autopsy case using the equation: $y^P = 34.6 + 0.55x$ [y^P: pancreas parenchymal volume (cubic centimeters), x: BMI].

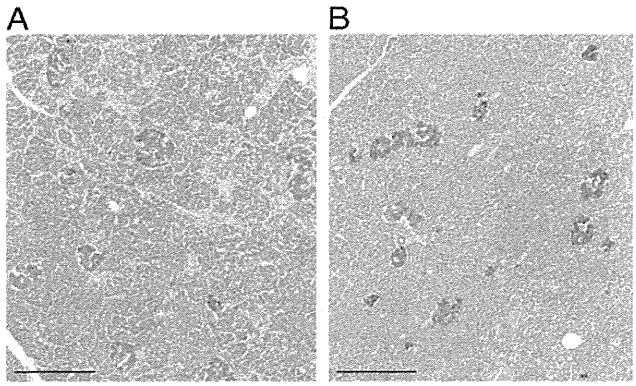

Figure 1. Representative photomicrographs of pancreas immunostained for insulin (brown) and with hematoxylin (×20 objective). No increase in β-cell area was observed in an obese individual (43 y old man, BMI 26.1 kg/m^2; B) compared with a lean individual (49 y old man, BMI 18.3 kg/m^2; A). Scale bar, 300 μm.

Assessment of β-cell mass

BCM was calculated as the product of %BCA, determined by immunohistochemical staining in each individual, and estimated pancreas parenchymal weight, determined as above (assuming 1 g of weight per 1 cm^3 pancreas volume).

Statistical analysis

Data are presented as mean ± SD in the text and tables and mean ± SEM in the figures. Statistical comparisons were carried out using a Student's *t* test, with a $P < .05$ taken as significant. Simple regression was carried out for correlation analysis.

Results

β-Cell mass in obesity

Representative pictures of the pancreas in lean and obese individuals are shown in Figure 1. The %BCA in obese subjects was significantly decreased compared with that in lean subjects (1.14 ± 0.81 vs 1.57 ± 0.84%, $P = .03$, Figure 2A). There was a significant negative correlation between β-cell area and BMI (r = −0.25, $P = .04$, Figure 2B), although the range of β-cell area largely overlapped between lean and obese subjects. Based on the population data, the pancreas parenchymal volume was estimated to be approximately 10% greater in obese subjects than in lean subjects (50.3 ± 2.2 vs 45.8 ± 1.0 cm^3, $P < .01$). Despite the greater pancreas volume in obese subjects, BCM, which was a product of %BCA, and the estimated pancreas parenchymal volume (assuming 1 g of weight per 1 cm^3 pancreas parenchymal volume) remained lower in obese subjects than in lean subjects, although the difference was not statistically significant (0.57 ± 0.40 vs 0.72 ± 0.38 g, $P = .12$, Figure 2C). There was no significant correlation between BCM and BMI (r = −0.16, $P = .19$, Figure 2D).

To exclude possible confounding factors, we conducted several subanalyses. After excluding subjects who died from malignancy, we confirmed no increase in %BCA and BCM in obese compared with lean subjects (n = 29, 0.80 ± 0.12 vs 1.63 ± 0.30%, $P = .01$ and 0.40 ± 0.06 vs 0.74 ± 0.14 g, $P = .03$, respectively). Similarly, after excluding subjects whose samples were obtained from the pancreatic head, there was no increase in %BCA and BCM in obese compared with lean subjects (n = 65, 1.13 ± 0.13 vs 1.63 ± 0.14%, $P = .01$, and 0.57 ± 0.07 vs 0.74 ± 0.06 g, $P = .06$). Finally, the results were also the same in subjects aged 60 years or less (n = 66, %BCA: 1.16 ± 0.15 vs 1.52 ± 0.14%, $P = .08$, and BCM: 0.58 ± 0.08 vs 0.70 ± 0.06 g, $P = .25$, in obese vs lean subjects, respectively). There was no correlation between age and %BCA or BCM (r = −0.21, $P = .09$ and r = −0.12, $P = .34$).

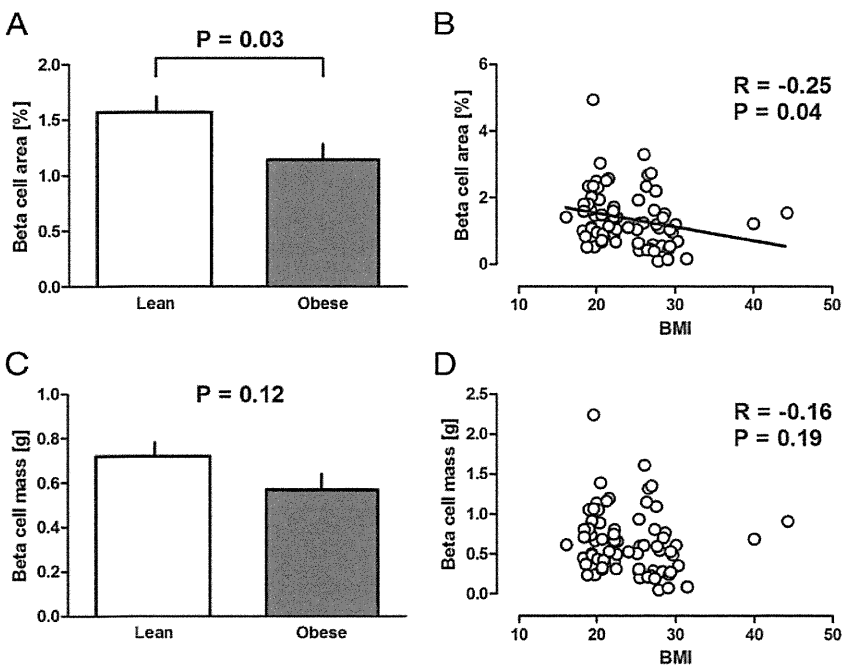

Figure 2. The %BCA and BCM in relation to obesity in Japanese individuals. The %BCA was significantly decreased in obese subjects compared with lean subjects (A), and there was a significant negative correlation between %BCA and BMI (B). After adjustment for pancreas volume, the estimated BCM in obese subjects was not significantly different from that in lean subjects (C). There was no significant correlation between BCM and BMI (D).

β-Cell turnover in obesity

Individual β-cell size determined as β-cell diameter was not significantly different between lean and obese subjects (8.53 ± 0.72 vs

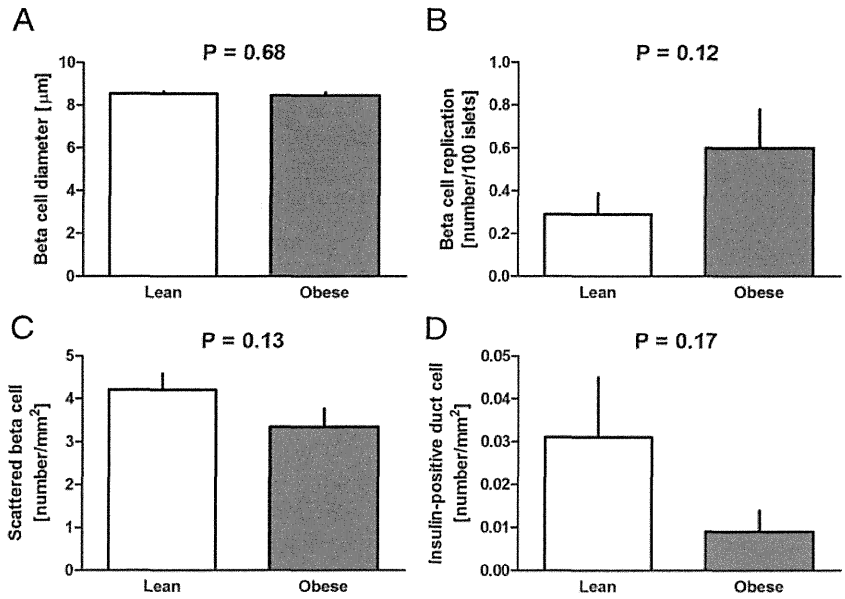

Figure 3. Comparisons of individual β-cell diameter (A), β-cell replication (B), scattered β-cells (C), and insulin-positive duct cells (D) between lean and obese subjects. None of them was significantly changed in obese subjects compared with lean subjects.

8.45 ± 0.79 μm, $P = .68$, Figure 3A). There was no significant difference in β-cell replication between the groups (0.29 ± 0.61 vs 0.60 ± 1.01 per 100 islets, $P = .12$, Figure 3B). Neither scattered β-cells nor insulin-positive duct cells were increased in obese subjects compared with lean subjects (3.4 ± 2.5 vs 4.2 ± 2.4/mm^2, $P = .13$ and $P = .009$ ± 0.029 vs 0.031 ± 0.088/mm^2, $P = .17$, respectively, Figure 3, C and D). β-cell apoptosis (ie, ssDNA positive β-cell) was not detected in either group. The rarity of the β-cell apoptosis was also confirmed by immunostaining for cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase-1 (data not shown).

α-Cell to β-cell ratio

Similarly to β cell area, there was no significant increase in %ACA in obese compared to lean subjects (0.79 ± 0.67 vs 1.06 ± 0.75%, $P = .14$, Figure 4A). The ratio of %ACA to %BCA was not significantly different between the 2 groups (0.91 ± 1.09 vs 0.75 ± 0.57, $P = .47$, Figure 4B). There was a significant positive correlation between the %ACA and the %BCA ($r = 0.46$, $P < .01$, Figure 5).

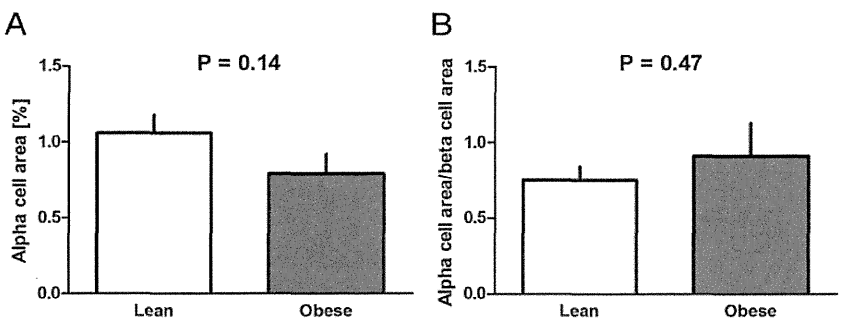

Figure 4. The %ACA (A) and the ratio of %ACA to %BCA (B) in lean and obese subjects are shown. There was no significant difference in %ACA and the ratio of %ACA to %BCA between lean and obese subjects.

Discussion

In this study we demonstrated that in Japanese nondiabetic obese individuals, we found the following: 1) there was no significant increase in BCM, 2) there was no detectable change in β-cell turnover, ie, β-cell replication, β-cell apoptosis, and β-cell neogenesis, and 3) there was no significant change in α-cell to β-cell ratio compared with that in lean individuals.

Type 2 diabetes is characterized by a deficit of BCM (1–4). However, the physiological change in BCM in humans remains largely unknown because of the difficulty accessing the pancreas. To date, BCM can be reasonably measured only by immunohistochemical analysis of the pancreas.

Obesity is an established risk factor for type 2 diabetes (17–19). Insulin sensitivity is decreased in the face of obesity, and insulin secretion is increased to compensate for this decreased insulin sensitivity to maintain normal glucose tolerance (7, 20, 21). However, there are limited data available as to the change in BCM in the presence of obesity in humans. It has been reported that BCM is significantly increased in obese individuals compared with lean individuals, and there is a significant positive correlation between BMI and BCM (2, 4, 8). To our knowledge, however, there are no data available in the Japanese population.

In this study, unexpectedly, BCM was not significantly increased in obese subjects compared with lean subjects. Because we used estimated pancreas volume to assess BCM, this might have affected estimated BCM in this study. However, most importantly, %BCA was significantly decreased in obese subjects compared with lean subjects, which is a striking difference from previous reports in the Caucasian population showing a significant increase in %BCA in obese individuals (8).

To exclude possible confounding factors that could affect BCM, we

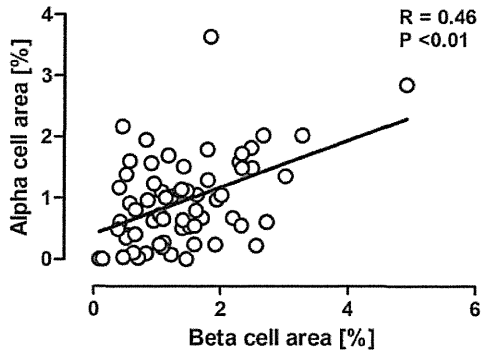

Figure 5. Correlation between %ACA and %BCA. There was a significant positive correlation between α-cell and β-cell area.

performed several subanalyses. It has been reported that the proportion of β-cells is different in the ventral portion of the pancreas head compared with other parts of the pancreas (3). However, we confirmed consistent results in a subanalysis excluding samples obtained from the pancreas head. It has been reported that the pancreas volume starts to decline after 60 years of age (16). Because of the difficulty of obtaining eligible cases, we included subjects aged 20–69 years in this study. Thus, the use of the equation for estimating pancreas volume in those aged older than 60 years might affect our findings. However, the results were consistent in the subanalysis excluding those aged older than 60 years.

We also observed that there was no significant change in β-cell turnover, ie, β-cell replication, β-cell neogenesis, and β-cell apoptosis. We used surrogate markers (ie, scattered β-cells and insulin positive duct cells) to assess β-cell neogenesis because it is not possible to directly assess β-cell neogenesis in humans (1, 22). BCM is regulated by input (ie, β-cell replication and neogenesis) and output (ie, mostly apoptosis) of β-cells (23–25). Butler et al (22) reported a significant (~50%) increase in BCM in pregnant women, accompanied by an increase in small islets and scattered β-cells, suggesting that β-cell neogenesis may contribute to the increase in BCM during pregnancy. Thus, the absence of a significant change in β-cell turnover in obese subjects in this study is also suggestive of no change in BCM in Japanese obese individuals, although it is possible that we were not able to detect any change in β-cell turnover in the obese subjects because the change in insulin sensitivity with obesity is more gradual compared with that with pregnancy.

An absence of increase in BCM in obese subjects was also confirmed by assessing the ratio of α-cell area to β-cell area. It has been reported that α-cell mass and α-cell to β-cell ratio are increased in subjects with type 2 diabetes (2, 3, 26). Whether α-cell to β-cell ratio is changed in the presence of obesity would be of interest. Henquin and Rahier (27) have reported that α-cell mass was not increased in obese subjects compared with lean subjects. In the present study, there was no increase in %ACA in obese subjects, and the ratio of α-cell to β-cell area was not significantly changed in those subjects. Thus, our and previous studies strongly suggest that there is no increase in α-cell mass in the presence of obesity.

Taken together, our findings consistently suggested that BCM was not increased in Japanese nondiabetic obese subjects compared with lean subjects. Although this result is distinct from those of previous reports, most of those studies have been conducted in Caucasian populations (4, 8). There has been one study from Korea reporting a positive correlation between %BCA and BMI; however, the positive correlation was seen in only a small number of nondiabetic subjects (n = 9), and the correlation between BCM and BMI was not reported (2). Recent studies have revealed that there is ethnic variability in the pathophysiology of type 2 diabetes. It is now widely appreciated that Asian populations with type 2 diabetes are leaner than Caucasian populations (28, 29). However, the underlying mechanism of this difference remains unknown. One possible explanation is that there is less β-cell functional capacity in Asians compared with Caucasians (9–11). An increase in insulin secretion in the face of obesity has also been shown in the Japanese population, but the degree of increment may be limited (30, 31). Our findings suggest that lower β-cell functional capacity in Asians may be associated with less β-cell regenerative capacity in response to obesity.

As with other autopsy studies, our study was not free of limitations. First, the cause of death or treatment prior to death might have affected BCM in our study population. Also, we did not have information on the changes in nutritional status or body weight prior to death. However, this should affect both the lean and obese groups. Likewise, illness prior to death, especially malignancy, might cause a loss of body weight at the time of death, which might result in underestimation of BMI. However, because the mean β-cell area of the lean subjects in this study was comparable with that of Caucasians (8), underestimation of BMI was unlikely to be the cause of the comparable BCM in lean and obese subjects observed in this study. We also confirmed our findings in a subanalysis of subjects without malignancy in whom the change in nutritional status prior to death was relatively small.

Mean BMI of obese subjects in this study was lower than that in previous studies in Caucasian populations (4, 8). It can be speculated that the undetectable change in BCM in this study was because of less adiposity in this study population. Although we were not able to assess insulin sensitivity in our study population, it has been reported that Asian people often have a greater degree of

visceral adiposity and higher risk of type 2 diabetes for the same BMI (28, 29, 32, 33), indicating that insulin sensitivity deteriorates at a lower BMI in Japanese compared with Caucasians. Thus, it is likely that the obese subjects in our study had lower insulin sensitivity compared with the lean subjects. Furthermore, previous studies have demonstrated a positive linear correlation between BMI and β-cell area or mass in the range of BMI of our study population (4, 8). In contrast, the correlation between β-cell area and BMI in this study rather tended to be negative. It is possible that BCM may increase in Japanese with more severe obesity comparable with that in Caucasians (ie, BMI $\geq$ 30 kg/m^2), although individuals with BMI $\geq$ 30 kg/m^2 comprise only approximately 2% of the Japanese population (12).

Although we obtained information on the presence of diabetes from medical records, if there were more subjects with undiagnosed diabetes in the obese group than in the lean group, this might result in underestimation of BCM in obese subjects. However, in the subjects in whom we were able to obtain glycated hemoglobin (HbA1c) level within 1 year prior to death (n = 38), none was apparently diabetic [ie, HbA1c $\geq$ 6.5% and random plasma glucose $\geq$ 200 mg/dL (34)] and there was no significant difference in HbA1c level between obese and lean subjects [5.2 $\pm$ 0.5 vs 5.4 $\pm$ 0.8%, $P = .41$, data are expressed as the National Glycohemoglobin Standardization Program value (35)]. Even in the subgroup of subjects whose HbA1c levels were less than 6.0% (n = 31), there was no increase in %BCA and BCM in obese subjects compared with lean subjects (1.16 $\pm$ 0.74 vs 1.92 $\pm$ 1.12%, $P = .03$, and 0.59 $\pm$ 0.37 vs 0.87 $\pm$ 0.51 g, $P = .14$, respectively).

Finally, although we carefully chose a similar study design to that of the previous study in a Caucasian population (8), it should be noted that our findings cannot be directly compared with the findings reported in that study. Technically, the use of a different scanning device or antibodies and the involvement of different investigators might have affected the different findings between the 2 studies. Moreover, because the subjects had been selected from different institutions, a difference in background characteristics of the study populations between the studies may exist. Thus, our findings should be confirmed in another Asian cohort. On the other hand, although we used the same equation to estimate pancreas parenchymal volume, the equation was estimated in a cohort in which the majority were Caucasian (16). Therefore, if the change in pancreas volume in Japanese differs from that in Caucasians, this might have affected our findings. However, a comparable change in pancreas mass or volume with obesity in Japanese has been reported in both anatomical (36) and imaging studies (37). In our previous study mentioned above (16), we also confirmed a similar change in pancreas parenchymal volume with obesity in Asians compared with Caucasians (Saisho, Y., unpublished data). Nonetheless, future investigations to clarify the effect of obesity on pancreas mass in the Japanese population are clearly warranted.

In this study, %BCA was decreased in obese subjects, and there was a significant negative correlation between %BCA and BMI. Based on this finding, one might assume that BCM decreased with obesity in Japanese. However, because of the facts that the significance of the difference between obese and lean subjects disappeared after adjusting for pancreas volume and that there was not an increase in apoptosis, a decrease in β-cell replication, or a decrease in neogenesis in obese subjects, we assume this possibility is unlikely and further studies are needed to address this question.

In conclusion, there was no increase in BCM and no detectable change in β-cell turnover in Japanese nondiabetic obese individuals. Our findings suggest the possibility that β-cell regenerative capacity in Japanese may differ from that in the Caucasian population. To test this hypothesis, we encourage further studies on BCM in different Asian cohorts, and if our findings are confirmed, direct comparison among different ethnicities will be warranted. Further comparison of islet morphology among different ethnic groups may provide an insight into the mechanism of β-cell regeneration in humans.

Acknowledgments

We thank Yuko Madokoro for technical assistance and Dr Wendy Gray for editing the manuscript. The authors are grateful to Professor Peter C. Butler for his constructive suggestions.

The authors made the following contributions: YS and HI conceived and designed the experiments: K.K., Y.S., and T.Y. performed the experiments; K.K. and Y.S. analyzed the data; K.K., Y.S., S.S., T.Y., and H.I. contributed to the discussion; K.K. and Y.S. wrote the manuscript; and K.K., Y.S., S.S., T.Y., and H.I. reviewed/edited the manuscript.

Address all correspondence and requests for reprints to: Yoshifumi Saisho, MD, PhD, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160–8582, Japan. E-mail: ysaisho@z5.keio.jp.

This work was supported by funding from the Nateglinide Memorial Toyoshima Research and Education Fund and the Daiwa Securities Health Foundation.

Disclosure Summary: The authors have no conflict of interest.

References

1. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes*. 2003;52:102–110.

2. **Yoon KH, Ko SH, Cho JH, et al.** Selective β-cell loss and α-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab*. 2003;88:2300–2308.
3. **Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S.** Reduced β-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia*. 2002;45:85–96.
4. **Rahier J, Guiot Y, Goebbels RM, Sempoux C, Henquin JC.** Pancreatic β-cell mass in European subjects with type 2 diabetes. *Diabetes Obes Metab*. 2008;10(suppl 4):32–42.
5. **Butler AE, Galasso R, Meier JJ, Basu R, Rizza RA, Butler PC.** Modestly increased β-cell apoptosis but no increased β-cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. *Diabetologia*. 2007;50:2323–2331.
6. **Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC.** Sustained β-cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? *Diabetologia*. 2005;48:2221–2228.
7. **Polonsky KS, Given BD, Van Cauter E.** Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. *J Clin Invest*. 1988;81:442–448.
8. **Saisho Y, Butler AE, Manesso E, Elashoff D, Rizza RA, Butler PC.** β-Cell mass and turnover in humans: effects of obesity and aging. *Diabetes Care*. 2013;36:111–117.
9. **Jensen CC, Cnop M, Hull RL, Fujimoto WY, Kahn SE.** β-Cell function is a major contributor to oral glucose tolerance in high-risk relatives of four ethnic groups in the US. *Diabetes*. 2002;51:2170–2178.
10. **Oka R, Yagi K, Sakurai M, et al.** Insulin secretion and insulin sensitivity on the oral glucose tolerance test (OGTT) in middle-aged Japanese. *Endocr J*. 2012;59:55–64.
11. **Heianza Y, Arase Y, Fujihara K, et al.** High normal HbA(1c) levels were associated with impaired insulin secretion without escalating insulin resistance in Japanese individuals: the Toranomon Hospital Health Management Center Study 8 (TOPICS 8). *Diabet Med*. 2012;29:1285–1290.
12. **Kanazawa M, Yoshiike N, Osaka T, Numba Y, Zimmet P, Inoue S.** Criteria and classification of obesity in Japan and Asia-Oceania. *Asia Pac J Clin Nutr* 2002;8(suppl 11):S732–S737.
13. **Naruse I, Keino H, Kawarada Y.** Antibody against single-stranded DNA detects both programmed cell death and drug-induced apoptosis. *Histochemistry*. 1994;101:73–78.
14. **Kawarada Y, Miura N, Sugiyama T.** Antibody against single-stranded DNA useful for detecting apoptotic cells recognizes hexadeoxynucleotides with various base sequences. *J Biochem*. 1998;123:492–498.
15. **Watanabe I, Toyoda M, Okuda J, et al.** Detection of apoptotic cells in human colorectal cancer by two different in situ methods: antibody against single-stranded DNA and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) methods. *Jpn J Cancer Res*. 1999;90:188–193.
16. **Saisho Y, Butler AE, Meier JJ, et al.** Pancreas volumes in humans from birth to age one hundred taking into account sex, obesity, and presence of type-2 diabetes. *Clin Anat*. 2007;20:933–942.
17. **Colditz GA, Willett WC, Rotnitzky A, Manson JE.** Weight gain as a risk factor for clinical diabetes mellitus in women. *Ann Intern Med*. 1995;122:481–486.
18. **Field AE, Coakley EH, Must A, et al.** Impact of overweight on the risk of developing common chronic diseases during a 10-year period. *Arch Intern Med*. 2001;161:1581–1586.
19. **Zimmet P, Alberti KG, Shaw J.** Global and societal implications of the diabetes epidemic. *Nature*. 2001;414:782–787.
20. **Bergman RN, Phillips LS, Cobelli C.** Physiologic evaluation of factors controlling glucose tolerance in man: measurement of insulin sensitivity and β-cell glucose sensitivity from the response to intravenous glucose. *J Clin Invest*. 1981;68:1456–1467.
21. **Defronzo RA.** Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes*. 2009;58:773–795.
22. **Butler AE, Cao-Minh L, Galasso R, et al.** Adaptive changes in pancreatic β-cell fractional area and β-cell turnover in human pregnancy. *Diabetologia*. 2010;53:2167–2176.
23. **Butler PC, Meier JJ, Butler AE, Bhushan A.** The replication of β-cells in normal physiology, in disease and for therapy. *Nat Clin Pract Endocrinol Metab*. 2007;3:758–768.
24. **Manesso E, Toffolo GM, Saisho Y, et al.** Dynamics of β-cell turnover: evidence for β-cell turnover and regeneration from sources of β-cells other than β-cell replication in the HIP rat. *Am J Physiol Endocrinol Metab*. 2009;297:E323–E330.
25. **Bonner-Weir S, Li WC, Ouziel-Yahalom L, Guo L, Weir GC, Sharma A.** β-Cell growth and regeneration: replication is only part of the story. *Diabetes*. 2010;59:2340–2348.
26. **Rahier J, Goebbels RM, Henquin JC.** Cellular composition of the human diabetic pancreas. *Diabetologia*. 1983;24:366–371.
27. **Henquin JC, Rahier J.** Pancreatic α-cell mass in European subjects with type 2 diabetes. *Diabetologia*. 2011;54:1720–1725.
28. **Hsu WC, Boyko EJ, Fujimoto WY, et al.** Pathophysiologic differences among Asians, native Hawaiians, and other Pacific Islanders and treatment implications. *Diabetes Care*. 2012;35:1189–1198.
29. **Sone H, Ito H, Ohashi Y, Akanuma Y, Yamada N.** Obesity and type 2 diabetes in Japanese patients. *Lancet*. 2003;361:85.
30. **Takahara M, Katakami N, Kaneto H, Noguchi M, Shimomura I.** Pancreatic β-cell function in lean and obese Japanese with various degrees of glucose tolerance [published online March 16, 2013]. *Endocr J*. doi:10.1507/endocrj.EJ13–0046.
31. **Saisho Y, Miyakoshi K, Tanaka M, et al.** β-Cell dysfunction and its clinical significance in gestational diabetes. *Endocr J*. 2010;57:973–980.
32. **Gill TP.** Cardiovascular risk in the Asia-Pacific region from a nutrition and metabolic point of view: abdominal obesity. *Asia Pac J Clin Nutr*. 2001;10:85–89.
33. **Kadowaki T, Sekikawa A, Murata K, et al.** Japanese men have larger areas of visceral adipose tissue than Caucasian men in the same levels of waist circumference in a population-based study. *Int J Obes (Lond)*. 2006;30:1163–1165.
34. **The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus.** Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. *J Diabetes Invest*. 2010;1:212–228.
35. **Committee on the Standardization of Diabetes Mellitus-Related Laboratory Testing of Japan Diabetes Society.** International clinical harmonization of glycated hemoglobin in Japan: from Japan Diabetes Society to National Glycohemoglobin Standardization Program values. *Diabetol Int*. 2012;3:8–10.
36. **Ogiu N, Nakamura Y, Ijiri I, Hiraiwa K, Ogiu T.** A statistical analysis of the internal organ weights of normal Japanese people. *Health Phys*. 1997;72:368–383.
37. **Goda K, Sasaki E, Nagata K, Fukai M, Ohsawa N, Hahafusa T.** Pancreatic volume in type 1 and type 2 diabetes mellitus. *Acta Diabetol*. 2001;38:145–149.

Loss of Pdk1-Foxo1 Signaling in Myeloid Cells Predisposes to Adipose Tissue Inflammation and Insulin Resistance

Yoshinaga Kawano,[1] Jun Nakae,[1] Nobuyuki Watanabe,[2] Shiho Fujisaka,[3] Kristy Iskandar,[4] Risa Sekioka,[1] Yoshitake Hayashi,[5] Kazuyuki Tobe,[3] Masato Kasuga,[6] Tetsuo Noda,[7] Akihiko Yoshimura,[8] Masafumi Onodera,[2] and Hiroshi Itoh[1]

Chronic inflammation in adipose tissue contributes to obesity-related insulin resistance. The 3-phosphoinositide-dependent protein kinase 1 (Pdk1)/forkhead transcription factor (Foxo1) pathway is important in regulating glucose and energy homeostasis, but little is known about this pathway in adipose tissue macrophages (ATMs). To investigate this, we generated transgenic mice that carried macrophage/granulocyte-specific mutations, including a *Pdk1* knockout (*LysMPdk1*$^{-/-}$), a *Pdk1* knockout with transactivation-defective Foxo1 (*Δ256LysMPdk1*$^{-/-}$), a constitutively active nuclear (CN) Foxo1 (*CNFoxo1*^{LysM}), or a transactivation-defective Foxo1 (*Δ256Foxo1*^{LysM}). We analyzed glucose metabolism and gene expression in ATM populations isolated with fluorescence-activated cell sorting. The *LysMPdk1*$^{-/-}$ mice exhibited elevated M1 macrophages in adipose tissue and insulin resistance. Overexpression of transactivation-defective Foxo1 rescued these phenotypes. *CNFoxo1*^{LysM} promoted transcription of the C-C motif chemokine receptor 2 (*Ccr2*) in ATMs and increased M1 macrophages in adipose tissue. On a high-fat diet, *CNFoxo1*^{LysM} mice exhibited insulin resistance. *Pdk1* deletion or Foxo1 activation in bone marrow–derived macrophages abolished insulin and interleukin-4 induction of genes involved in alternative macrophage activation. Thus, Pdk1 regulated macrophage infiltration by inhibiting Foxo1-induced *Ccr2* expression. This shows that the macrophage Pdk1/Foxo1 pathway is important in regulating insulin sensitivity in vivo. ***Diabetes* 61:1935–1948, 2012**

Obesity is a predisposing factor for the development of type 2 diabetes, hypertension, hyperlipidemia, and atherosclerosis (1). Chronic activation of intracellular proinflammatory pathways in adipose tissue contributes to obesity-related insulin resistance. Adipose tissue macrophages (ATMs) are a major source of proinflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α, which can decrease insulin sensitivity in insulin target cells (2). However, only sparse evidence suggests that ATMs may become insulin resistant and play a role in insulin signaling (3–9).

The 3-phosphoinositide-dependent protein kinase 1 (Pdk1)–forkhead transcription factor (Foxo1) signaling pathway regulates energy and glucose metabolism in several insulin-responsive tissues, including pancreatic β-cells and proopiomelanocortin and agouti-related protein neurons (10,11). However, few studies investigate this signaling pathway in ATMs. Recent reports suggest that activation of Foxo1 in macrophages promotes inflammation by inducing IL-1β expression (12) or toll-like receptor 4–mediated signaling (13). They show that Foxo1 could induce inflammatory cascades, but they do not investigate the role of Foxo1 specifically in ATMs in vivo.

In the current study, we generated transgenic mice that carried macrophage-specific mutations, including a *Pdk1* knockout, a constitutively nuclear (CN) *Foxo1*, or a transactivation-defective *Foxo1*. We analyzed insulin sensitivity in these mice in vivo. We found a novel Pdk1-Foxo1 signaling mechanism that regulated M1 macrophage recruitment.

RESEARCH DESIGN AND METHODS

Mice. All experimental protocols with mice were approved by the animal ethics committees of the Keio University School of Medicine (09134-1). To create macrophage-specific *Pdk1* knockout mice, *Pdk1*$^{flox/flox}$ mice (11) were crossed with *LysMCre* transgenic mice (14). The generation of *R26*^{floxneoCNFoxo1} mice was described previously (11). Only animals from the same generation of the mixed-background strain were compared. All mice studied were examined on a B6/129 mixed genetic background. Mice were obtained from two independent cohorts of independent breeders, and littermates were used for every in vivo study. Animals were housed in sterile cages in a barrier animal facility at 22–24°C with a 12-h light/dark cycle.

Antibodies. All antibodies used in the current study are available upon request.

Analytical procedures. For high-fat diet (HFD) studies, we used age-matched (28-week-old) mice. We started the HFD at age 4 weeks for the 24-week HFD and at age 24 weeks for the 4-week HFD. All of the HFD mice were compared with age-matched mice fed a normal chow diet (NCD). The HFD was described previously (15). Analysis was limited to male mice because they are more susceptible to insulin resistance and diabetes. We performed intraperitoneal glucose tolerance tests (IPGTTs) after an overnight fast and insulin tolerance tests (ITTs) after fasting for 3–5 h. The area under the curve (AUC) was calculated from the level of each measured point by the trapezoidal method.

Flow cytometric analysis. Flow cytometric analysis was performed as described previously (16).

Hepatic glycogen content. We measured glycogen content as described previously (17).

Immunofluorescence. Double-positive cells were counted and marked digitally to prevent multiple counts with Adobe Photoshop CS4 EXTENDED and ImageJ software (National Institutes of Health, Bethesda, MD). Cells were counted in eight mice for each HFD duration. At least 300 cells were counted in each mouse.

From [1]Frontier Medicine on Metabolic Syndrome, Division of Endocrinology, Metabolism, and Nephrology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; the [2]Department of Human Genetics, National Center for Child Health and Development, Tokyo, Japan; the [3]Department of Internal Medicine, University of Toyama, Toyama, Japan; the [4]Pediatric Research Office, Department of Child Health, Faculty of Medicine, Universitas Gadjah Mada, Sardjito Hospital, Yogyakarta, Indonesia; the [5]Division of Molecular Medicine and Medical Genetics, International Center for Medical Research and Treatment, Kobe University Graduate School of Medicine, Kobe, Japan; the [6]Research Institute, International Medical Center of Japan, Tokyo, Japan; the [7]Department of Cell Biology, Japanese Foundation for Cancer Research, Cancer Institute, Tokyo, Japan; and the [8]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan.

Corresponding author: Jun Nakae, jnakae35@sc.itc.keio.ac.jp.

Received 6 June 2011 and accepted 6 March 2012.

DOI: 10.2337/db11-0770

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-0770/-/DC1.

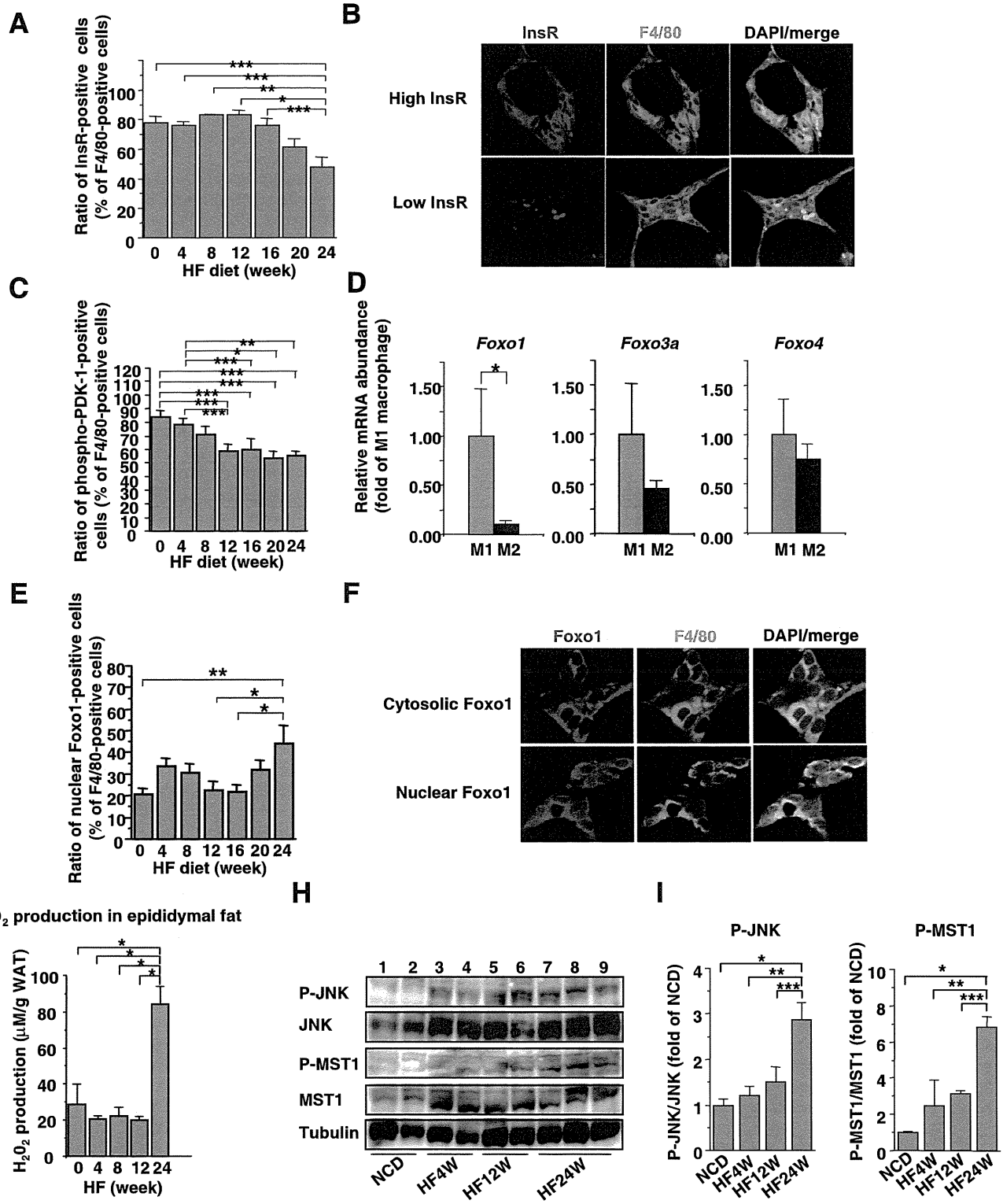

FIG. 1. Characterization of InsR, Pdk1, and Foxo1 in ATMs during an HFD. *A*: The percentages of InsR$^+$ cells among F4/80$^+$ cells in epididymal fat from age-matched wild-type mice fed an NCD and 24 weeks of an HFD. Values are means + SEM of eight mice. *P < 0.005, **P < 0.01, and ***P < 0.05 (one-factor ANOVA). *B*: Representative immunofluorescence images of epididymal fat double labeled for InsR and F4/80 in wild-type mice fed an HFD for 24 weeks. Cells that exhibit high expression level of InsR protein (*top*); cells that exhibit low or faint expression level of InsR (*bottom*). Red, green, and blue indicate InsR, F4/80, and DAPI staining, respectively. *C*: The percentages of phospho-PDK1$^+$ cells among F4/80$^+$ cells in epididymal fat from age-matched wild-type mice fed an NCD and 24 weeks of an HFD. Values are means + SEM of eight mice. *P < 0.005, **P < 0.01, ***P < 0.05 (one-factor ANOVA). *D*: Real-time PCR analysis of *Foxo* family members in cell populations sorted by flow cytometric analysis of the SVF from the epididymal fats of wild-type mice fed an HFD for 16 weeks, using anti-F4/80, anti-CD11c, and anti-CD206 antibodies. The levels of each transcript were normalized to the level in M1 macrophages. Values are means + SEM of three mice. *P < 0.05 (one-factor ANOVA, M1 vs. M2 macrophages). *E*: The percentages of nuclear Foxo1$^+$ cells among F4/80$^+$ cells in the epididymal fat of age-matched wild-type

H_2O_2 production. Measurement of H_2O_2 production was performed as described elsewhere (18). Epididymal fat was dissected from age-matched male C57BL/6J mice on either an NCD or a 4-24 week HFD.

Counting crown-like structures. Measurement of number of crown-like structures (CLSs) was performed as described previously (16).

Cell size measurements. Adipocyte size was measured with FLVFS-LS software (Flovel, Tokyo, Japan) by manually tracing a minimum of 1,200 adipocytes for each mouse. We measured adipocytes in at least six mice of each genotype.

Isolation of murine bone marrow–derived macrophages. Isolation of bone marrow–derived macrophages (BMDMs) was performed as described elsewhere (19).

Transwell migration assay. Transwell migration assays were performed as previously described (20).

Viral transduction. Adenovirus constructs that encoded Foxo1 mutants are described elsewhere (21,22). RAW264.7 cells were infected with adenoviruses (10–100 multiplicity of infection [MOI]) and harvested after 48 h. For cotransductions, cells were first transduced with an adenovirus that encoded Flag-CNFoxo1 at the indicated MOI for 8 h. The virus was then removed from the culture dish, and the cells were transduced with another adenovirus that encoded HA-Δ256Foxo1 at the indicated MOI for 8 h.

RNA isolation and real-time PCR. The isolation of total RNA and real-time PCR were performed as described previously (15). All primer sequences are available upon request.

Western blotting. Western blotting was performed as described previously (15). Insulin-stimulated phosphorylation of insulin receptor substrates (IRSs) and Akt were performed as described elsewhere (23).

Construction of C-C motif chemokine receptor 2 promoter–directed luciferase reporter vectors. Several DNA fragments containing the mouse C-C motif chemokine receptor 2 (*Ccr2*) promoter were PCR-amplified from mouse genomic DNA. After verifying their nucleotide sequences by DNA sequencing, the *Ccr2* promoter fragments were cloned into the luciferase reporter pGL3-Basic vector (Promega, Madison, WI). All primer sequences are available upon request.

Site-directed mutagenesis. The QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to alter the consensus Foxo1 binding elements in the *Ccr2* promoter in PGL3-Basic vectors. Mutated nucleotides were confirmed with DNA sequencing. All primer sequences are available upon request.

Luciferase assay. The luciferase assay was performed as described previously (22).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) and the super shift assay were performed as described previously (24).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed as described previously (22).

Statistical analysis. We calculated descriptive statistics with ANOVA followed by Fisher test (Statview; SAS Institute Inc.). $P < 0.05$ was considered significant. Differences between two groups or among three groups were investigated with two-way repeated-measures ANOVA with an ad hoc multiple comparison method (Fisher least significant differences [LSD] test).

RESULTS

Insulin receptor expression and Pdk1 phosphorylation in ATMs during an HFD. To explore the significance of insulin signaling pathway in ATM, we examined insulin receptor (InsR) protein expression in ATMs by double immunofluorescence with anti-InsR and anti-F4/80 antibodies. During the HFD, ATM InsR protein levels were significantly reduced by ~50% compared with controls (Fig. 1*A* and *B*).

Next, we explored Pdk1 expression in ATMs under different diets. Immunofluorescence of epididymal fat from C57BL/6J mice on an HFD for 16 weeks revealed that cells positive for the macrophage marker CD68 were also positive for Pdk1 (Fig. 2*A*, *top*). Because Pdk1 activity depends on Ser 241 phosphorylation (25), we probed with an antiphospho-Pdk1 antibody. On an NCD, ~80% of F4/80$^+$ cells were stained with antiphospho-Pdk1. On an HFD for 24 weeks, the proportion of phospho-Pdk1$^+$ ATMs gradually decreased from 80% to from 40 to 50% (Fig. 1*C*). These data confirm that the InsR-Pdk1 pathway was functionally regulated in ATMs during the HFD.

Foxo1 in ATMs under an HFD. To explore the relative importance of Foxo family members in ATMs, we compared the expression of *Foxo1*, *Foxo3a*, and *Foxo4* in M1 and M2 macrophages isolated from the stromal vascular fraction (SVF) of epididymal fat from C57BL/6J mice fed an HFD for 16 weeks. We defined F4/80$^+$CD11c$^+$CD206$^-$ cells as M1 macrophages and F4/80$^+$CD11c$^-$CD206$^+$ cells as M2 macrophages (16). M1 macrophages showed significantly increased *Foxo1* expression compared with M2 macrophages. *Foxo3a* expression was also increased in M1 compared with M2 macrophages but not significantly. In contrast, M1 and M2 macrophages showed similar *Foxo4* expression (Fig. 1*D*). These observations suggest that Foxo1 played an essential role in ATMs.

Because Foxo1 activity depends on its subcellular localization (26), we examined Foxo1 with immunofluorescence in ATMs from age-matched C57BL/6J mice fed an NCD or HFD. Under the NCD, ~20% of Foxo1 was localized to the nucleus. After 24 weeks of an HFD, ~45% of Foxo1 was localized to the nucleus (Fig. 1*E* and *F*). These data suggest that Foxo1 was functionally significant in ATMs.

Foxo1 is regulated by oxidative stress through H_2O_2 production and the Jun NH_2-terminal kinase (JNK)–mammalian Ste20-like kinase 1 (MST1) pathway, which induces Foxo1 nuclear translocation (27–30). The production of H_2O_2 significantly increased at ~24 weeks of HFD (Fig. 1*G*). Furthermore, JNK and MST1 phosphorylation significantly increased after 24 weeks of HFD (Fig. 1*H* and *I*). These data suggest that both decreased Pdk1 phosphorylation and activation of the JNK-MST1 pathway may contribute to Foxo1 nuclear localization.

Deletion of Pdk1 in ATMs causes insulin resistance with rescue by transactivation-defective Foxo1. To clarify the function of Pdk1 in ATMs, we generated mice that lacked *Pdk1* in macrophages/granulocytes (*LysMPdk1*$^{-/-}$). Efficient, specific *Pdk1* deletion was evidenced by immunofluorescence (Fig. 2*A*) and Western blot analysis (Fig. 2*B*). Thus, we could study the effects of cell-specific *Pdk1* deficiency.

The deletion of *Pdk1* in ATMs was expected to cause nuclear localization of Foxo1. Immunofluorescence with an anti-Foxo1 antibody in epididymal fat revealed that ~60–70% of Foxo1 was localized to the nuclei of ATMs in *LysMPdk1*$^{-/-}$ mice (Fig. 2*C*). We assumed that Foxo1 was active in *Pdk1*-deficient ATMs and that this activity could be blocked with the dominant-negative form of Foxo1 (Δ256Foxo1), which lacked a COOH-terminal transactivation domain (31). To investigate this, we crossed

mice fed an NCD and 24 weeks of an HFD. Values are means + SEM of eight mice. *$P < 0.001$, **$P < 0.005$ (one-factor ANOVA). *F*: Representative immunofluorescence images of epididymal fat double labeled for Foxo1 and F4/80 in wild-type mice fed an HFD for 24 weeks. Cytosolic (*top*) and nuclear Foxo1 (*bottom*). Red, green, and blue indicate Foxo1, F4/80, and DAPI staining, respectively. *G*: The release of H_2O_2 from epididymal fats from age-matched male C57BL/6J mice fed an NCD or 4–24 weeks of an HFD. Values are expressed as mean ± SEM of five mice in each condition. *$P < 0.001$ (one-factor ANOVA). *H*: Western blotting of epididymal fats from age-matched male C57BL/6J mice fed an NCD or 4–24 weeks of an HFD. After transference to nylon membrane, tissue lysates (200 μg) were blotted to the indicated antibodies. *I*: Quantitative analysis of JNK and MST1 phosphorylation in epididymal fats. The intensity of each band was measured using NIH Image 1.62, and the intensities of bands of phospho-JNK or phospho-MST1 bands were corrected by total JNK or MST1 and calculated as the fold change from NCD. Data are means + SEM of five mice in each genotype. *$P < 0.001$, **$P < 0.005$, and ***$P < 0.05$ (one-factor ANOVA of NCD vs. HFD). WAT, white adipose tissue; P, phospho; W, weeks. (A high-quality digital representation of this figure is available in the online issue.)

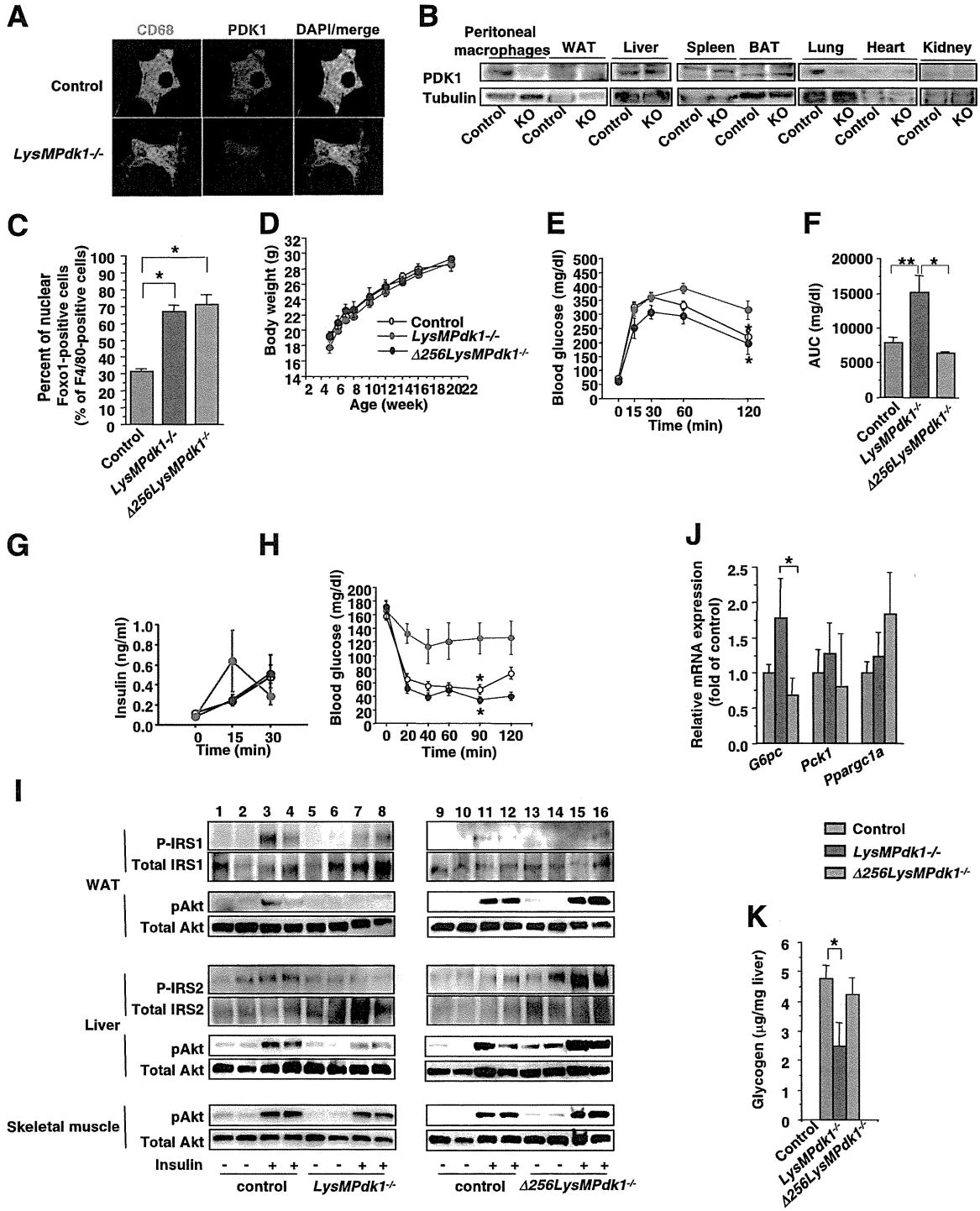

FIG. 2. Effects of the deletion of *Pdk1* or inhibition of the transactivation of Foxo1 on glucose metabolism and insulin sensitivity. *A*: Representative immunofluorescence images of epididymal fat double labeled for CD68 and PDK1 in 24-week-old wild-type and *LysMPdk1*$^{-/-}$ mice. Green, red, and blue indicate CD68, PDK1, and DAPI staining, respectively. *B*: Expression of Pdk1 in peritoneal macrophages and peripheral tissues. Western blot of Pdk1 and tubulin (loading control) in the white adipose tissue (WAT), liver, spleen, brown adipose tissue (BAT), lungs, heart, and kidneys of control and *LysMPdk1*$^{-/-}$ (KO) mice. *C*: The percentages of nuclear Foxo1^{+} among F4/80^{+} cells in epididymal fat of control, *LysMPdk1*$^{-/-}$, and *Δ256LysMPdk1*$^{-/-}$ mice aged 20–24 weeks. Counting of cells stained with anti-F4/80 and anti-FOXO1 are described in RESEARCH DESIGN AND METHODS. Values are means + SEM of three mice in each genotype. *P < 0.005 (one-factor ANOVA). *D*: Body weight of control, *LysMPdk1*$^{-/-}$, and *Δ256LysMPdk1*$^{-/-}$ fed an NCD. Data are means + SEM of 18–20 mice in each genotype. *E*: IPGTT of control (open circle), *LysMPdk1*$^{-/-}$ (red circle), and *Δ256LysMPdk1*$^{-/-}$ (blue circle) mice fed an NCD. Data are means + SEM of 20–25 mice in each genotype at age 20–24 weeks. *P < 0.05 (two-way repeated-measures ANOVA with an ad hoc multiple comparison method [Fisher LSD test] of *LysMPdk1*$^{-/-}$ vs. control or *Δ256LysMPdk1*$^{-/-}$ mice). *F*: Comparison of AUC in control, *LysMPdk1*$^{-/-}$, and *Δ256LysMPdk1*$^{-/-}$ mice during IPGTT. Data are means + SEM of 20–25 mice in each genotype. *P < 0.01 (two-way repeated-measures ANOVA with Fisher LSD test of *LysMPdk1*$^{-/-}$ vs. *Δ256LysMPdk1*$^{-/-}$ mice) and **P < 0.05 (two-way repeated-measures ANOVA with Fisher LSD test of *LysMPdk1*$^{-/-}$ vs. control mice). *G* and *H*: Insulin secretion (*G*) of control (open circle), *LysMPdk1*$^{-/-}$ (red circle), and *Δ256LysMPdk1*$^{-/-}$ (blue circle) mice during IPGTT and blood glucose (H) during ITT. Data are

R26^{floxneoΔ256FoxO1} (11) with *LysMCre* transgenic mice to generate *R26*^{floxneoΔ256FoxO1} *LysMCre* (*Δ256Foxo1*^{LysM}) double heterozygotes. Real-time PCR analysis and immunofluorescence confirmed the macrophage-specific expression of the transgene and the nuclear localization of FLAG-Δ256Foxo1, respectively (Supplementary Figs. 1 and 2). We crossed *Δ256Foxo1*^{LysM} with *Pdk1*$^{flox/+}$ to generate double mutant mice (*Δ256Foxo1*^{LysM}*Pdk1*$^{+/-}$). Finally, these mice were crossed with *Pdk1*$^{flox/+}$ to generate *Δ256Foxo1*^{LysM}*Pdk1*$^{-/-}$ (*Δ256LysMPdk1*$^{-/-}$) mice (Supplementary Fig. 3). As expected, *Δ256LysMPdk1*$^{-/-}$ mice showed excess nuclear Foxo1 in F4/80$^+$ cells from epididymal fat (Fig. 2*C*).

The *LysMPdk1*$^{-/-}$ and *Δ256LysMPdk1*$^{-/-}$ mice exhibited normal body weight when fed an NCD (Fig. 2*D*), and their epididymal fat tissue weight and adipocyte sizes were similar to those of control mice (Supplementary Fig. 4*A* and *B*). However, the IPGTTs revealed that *LysMPdk1*$^{-/-}$, but not *Δ256LysMPdk1*$^{-/-}$, mice exhibited glucose intolerance (Fig. 2*E* and *F*). Insulin secretion during the IPGTT was higher in *LysMPdk1*$^{-/-}$ mice than in controls and *Δ256LysMPdk1*$^{-/-}$ mice, but the difference was not significant (Fig. 2*G*). Furthermore, insulin tolerance significantly decreased in *LysMPdk1*$^{-/-}$ mice compared with control and *Δ256LysMPdk1*$^{-/-}$ mice (Fig. 2*H*). These data indicate that the deletion of *Pdk1* deteriorates insulin sensitivity and that the ectopic expression of Δ256Foxo1 ameliorates insulin sensitivity.

To identify the tissues that are responsible for insulin resistance, we investigated insulin-stimulated phosphorylation of IRS1, IRS2, and/or Akt in epididymal fats, liver, and skeletal muscle from control, *LysMPdk1*$^{-/-}$, and *Δ256LysMPdk1*$^{-/-}$ mice. In epididymal fat and liver, insulin-stimulated phosphorylation of IRS1 or IRS2 and Akt was significantly decreased in *LysMPdk1*$^{-/-}$ mice compared with control mice (Fig. 2*I*). However, insulin-stimulated phosphorylation of IRS and Akt in epididymal fat and liver from *Δ256LysMPdk1*$^{-/-}$ mice was similar to that of control mice (Fig. 2*I*). The expression of *G6pc* was significantly increased in liver from *LysMPdk1*$^{-/-}$ compared with *Δ256LysMPdk1*$^{-/-}$ mice (Fig. 2*J*); moreover, the hepatic glycogen content of *LysMPdk1*$^{-/-}$ mice was significantly decreased compared with control and *Δ256LysMPdk1*$^{-/-}$ mice (Fig. 2*K*). In contrast, Akt phosphorylation in skeletal muscle from *LysMPdk1*$^{-/-}$ mice was similar to that of control and *Δ256LysMPdk1*$^{-/-}$ mice (Fig. 2*I*). These data indicate that the deletion of *Pdk1* in ATMs led to insulin resistance, mainly in adipose tissue and liver, and that ectopic expression of Δ256Foxo1 ameliorated insulin resistance in those tissues.

Deletion of Pdk1 caused an increase of M1 macrophages in adipose tissues. A CLS is the accumulation of immune cells around dead adipocytes (32). We found that the number of F4/80$^+$ CLSs per field in epididymal fat was significantly higher in *LysMPdk1*$^{-/-}$ mice than in control and *Δ256LysMPdk1*$^{-/-}$ mice (Fig. 3*A*).

The SVF of adipose tissue from 20-week-old mice contained a substantially higher proportion of F4/80$^+$ cells in *LysMPdk1*$^{-/-}$ compared with control mice (Fig. 3*B* and *C*). Analysis of macrophage subpopulations in the SVF showed a higher proportion of F4/80$^+$CD11c$^+$CD206$^-$ cells in *LysMPdk1*$^{-/-}$ mice than in control mice (Fig. 3*B* and *C*). In contrast, the adipose tissue of *Δ256LysMPdk1*$^{-/-}$ mice showed significantly reduced proportions of F4/80$^+$ cells and F4/80$^+$CD11c$^+$CD206$^-$ cells compared with *LysMPdk1*$^{-/-}$ mice (Fig. 3*B* and *C*). These data suggest that the deletion of *Pdk1* caused a significant increase in the proportion of M1 macrophages in epididymal fat, and the proportion was reduced with the overexpression of Δ256Foxo1.

Consistent with the above findings, the expression of chemokine (C-C motif) ligand 2 (*Ccl2*) (also known as monocyte chemoattractant protein-1 [*Mcp-1*]) and *Cd68* in epididymal fat (Fig. 3*D*) and of *Ccr2* and *Tnfa* in SVF from *LysMPdk1*$^{-/-}$ mice were significantly increased compared with control and *Δ256LysMPdk1*$^{-/-}$ mice (Fig. 3*E*). Furthermore, the expression level of IL-1 receptor antagonist, which is a naturally occurring antagonist of IL-1β and produced by adipose and other tissues (33), in SVF from *LysMPdk1*$^{-/-}$ mice was significantly decreased compared with control mice (Fig. 3*E*). These data support the notion that the deletion of *Pdk1* increased the recruitment of M1 macrophages to adipose tissues.

Macrophage-specific CNFoxo1 transgenic (*CNFoxo1*^{LysM}) mice exhibited insulin resistance. To clarify the function of Foxo1 in ATMs, we generated macrophage-specific CNFoxo1 transgenic mice. We crossed *Rosa26-CNFoxo1* (11) with *LysMCre* (*CNFoxo1*^{LysM}) mice. Real-time PCR revealed that the transgene was expressed exclusively in the spleen, liver, hypothalamus, and lung and in ATMs from the epididymal fat (Supplementary Fig. 5). These tissues have tissue-specific macrophages, which include the cells in the sinusoidal lining of the spleen, Kupffer cells in the liver, microglia in the hypothalamus, and alveolar macrophages in the lung (14,34,35). Therefore, resident macrophages likely account for the increased expression of the transgene in these tissues. Immunofluorescence of the epididymal fat showed that FLAG-CNFoxo1 was exclusively localized in the nucleus of F4/80$^+$ macrophages (Supplementary Fig. 6). Furthermore, immunofluorescence revealed that ~50% of F4/80$^+$ cells in epididymal fat of *CNFoxo1*^{LysM} mice were positive for FLAG (Fig. 4*A*) and that the percentages of nuclear Foxo1$^+$ cells in adipose tissue of *CNFoxo1*^{LysM} fed an HFD for 16 weeks was significantly increased compared with control mice fed an HFD or *CNFoxo1*^{LysM} fed an NCD (Fig. 4*B*). These results show that *CNFoxo1*^{LysM} mice were an appropriate model for studying the specific effects of overexpressing Foxo1 in ATMs.

On an NCD, *CNFoxo1*^{LysM} mice exhibited normal body weight, glucose tolerance, insulin secretion, and insulin sensitivity (Supplementary Fig. 7*A–D*). On an HFD, the body

means + SEM of 20–25 mice in each genotype. *$P < 0.05$ (two-way repeated-measures ANOVA with Fisher LSD test of control vs. *LysMPdk1*$^{-/-}$ or *Δ256LysMPdk1*$^{-/-}$ mice). *I*: Insulin-stimulated phosphorylation of IRSs and Akt in epididymal fat (WAT), liver, and skeletal muscle from control, *LysMPdk1*$^{-/-}$, and *Δ256LysMPdk1*$^{-/-}$ mice. For Western blotting with phospho- and total Akt, the same filters, in which tissue lysates (200 μg) were transferred, were blotted with the indicated antibodies. For immunoprecipitation of IRSs, tissue lysates (10 mg) were immunoprecipitated with the indicated antibodies and blotted with anti-phosphotyrosine antibody and then reblotted with anti-IRS antibody. *J*: Expression of genes specific for gluconeogenesis in liver from control, *LysMPdk1*$^{-/-}$, and *Δ256LysMPdk1*$^{-/-}$ mice in the random fed state. Values were normalized to β-actin expression and represent means + SEM of 8–10 mice in each genotype. *$P < 0.05$ (one-factor ANOVA of *LysMPdk1*$^{-/-}$ vs. *Δ256LysMPdk1*$^{-/-}$). *K*: Hepatic glycogen content. Control ($n = 9$), *LysMPdk1*$^{-/-}$ ($n = 9$), and *Δ256LysMPdk1*$^{-/-}$ ($n = 8$) mice were killed in the random fed state for the determination of glycogen levels in liver extracts. Data are means + SEM of hepatic glycogen content corrected by the weight of liver per genotype. *$P < 0.05$ (one-factor ANOVA of control vs. *LysMPdk1*$^{-/-}$ mice). (A high-quality digital representation of this figure is available in the online issue.)