tion (A1330V) in the low-density lipoprotein receptor-related protein 5 gene (LRP5) with bone mineral density in adult Japanese women. *Bone* 40: 997–1005.

20. Ferrari SL, Deutsch S, Baudoin C, Cohen-Solal M, Ostertag A, Antonarakis SE, Rizzoli R, de Vernejoul MC (2005) LRP5 gene polymorphisms and idiopathic osteoporosis in men. LRP5 gene polymorphisms and idiopathic osteoporosis in men. *Bone* 37: 770–775.
21. Ferrari SL, Deutsch S, Choudhury U, Chevalley T, Bonjour JP, Dermitzakis ET, Rizzoli R, Antonarakis SE (2004) Polymorphisms in the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with variation in vertebral bone mass, vertebral bone size, and stature in whites. *Am J Hum Genet* 74: 866–875.
22. Guo YF, Xiong DH, Shen H, Zhao LJ, Xiao P, Guo Y, Wang W, Yang TL, Recker RR, Deng HW (2006) Polymorphisms of the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with obesity phenotypes in a large family-based association study. *J Med Genet* 43: 798–803.
23. Dessimoz J, Bonnard C, Huelsken J, Grapin-Botton A (2005) Pancreas-specific deletion of β-catenin reveals Wnt-dependent and Wnt-independent functions during development. *Curr Biol* 15: 1677–1683.
24. Heller RS, Dichmann DS, Jensen J, Miller C, Wong G, Madsen OD, Serup P (2002) Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. *Dev Dyn* 225: 260–270.
25. Rulifson IC, Karnik SK, Heiser PW, ten Berge D, Chen H, Gu X (2007) Wnt signaling regulates pancreatic beta cell proliferation. *Proc Natl Acad Sci USA* 104: 6247–6252.

Replication of Genome-Wide Association Studies of Type 2 Diabetes Susceptibility in Japan

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Background: In Europeans and populations of European origin, several groups have recently identified novel type 2 diabetes susceptibility genes, including *FTO, SLC30A8, HHEX, CDKAL1, CDKN2B,* and *IGF2BP2,* none of which were in the list of functional candidates.

Objective and Design: The aim of this study was to replicate in a Japanese population previously identified associations of single nucleotide polymorphisms (SNPs) within 10 candidate loci with type 2 diabetes using a relatively large sample size: 1921 subjects with type 2 diabetes and 1622 normal controls.

Results: A total of 15 SNPs were genotyped. Eight SNPs in five loci were found to be associated with type 2 diabetes: rs3802177 [odds ratio (OR) = 1.16 (95% confidence interval (CI) 1.05–1.27); $P = 4.5 \times 10^{-3}$] in *SLC30A8*; rs1111875 [OR = 1.27 (95% CI 1.14–1.40); $P = 1.4 \times 10^{-5}$] and rs7923837 [OR = 1.27 (95% CI 1.13–1.43); $P = 1.0 \times 10^{-4}$] in *HHEX*; rs10811661 [OR = 1.27 (95% CI 1.15–1.40); $P = 1.9 \times 10^{-6}$] in *CDKN2B*; rs4402960 [OR = 1.23 (95% CI 1.11–1.36); $P = 8.1 \times 10^{-5}$] and rs1470579 [OR = 1.18 (95% CI 1.07–1.31); $P = 8.3 \times 10^{-4}$] in *IGF2BP2*; and rs7754840 [OR = 1.28 (95% CI 1.17–1.41); $P = 4.5 \times 10^{-7}$] and rs7756992 [OR = 1.27 (95% CI 1.15–1.40); $P = 9.8 \times 10^{-7}$] in *CDKAL1*. The first and second strongest associations were found at variants in *CDKAL1 and CDKN2B*, both of which are involved in the regenerative capacity of pancreatic β-cells.

Conclusion: Some of these variants represent common type 2 diabetes-susceptibility genes in both Japanese and Europeans. (*J Clin Endocrinol Metab* **93: 3136–3141, 2008)**

Type 2 diabetes is a complex disease with several genes and environmental factors involved in onset and development. To date, a number of genes have been reported to be associated with type 2 diabetes. Most of these were investigated because of their assumed relevance to the pathogenesis of type 2 diabetes based on their functions. However, because the pathogenesis of

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Abbreviations: BMI, Body mass index; *CDKAL1*, cyclin-dependent kinase inhibitor 5 regulatory subunit associated protein 1-like 1 gene; *CDKN2B*, cyclin-dependent kinase inhibitor 2B gene; CI, confidence interval; *EXT2*, exostosin 2; *FTO*, fat mass and obesity associated gene; *GCKR*, glucokinase regulatory protein gene; *HHEX*, hematopoietically expressed homeobox gene; HOMA, homeostasis model assessment; HOMA-β, homeostasis model assessment of β-cell function; HOMA-IR, homeostasis model assessment of insulin resistance; *IGF2BP2*, IGF2 mRNA binding protein 2 gene; LD, linkage disequilibrium; OR, odds ratio; *SLC30A8*, zinc transporter gene; SNP, single nucleotide polymorphism; TG, triglyceride.

type 2 diabetes is yet to be elucidated completely, the candidate-gene approach is limited in power to detect novel disease-susceptibility genes. A strongly associated type 2 diabetes gene, transcription factor 7-like 2, has been identified by a genome-wide linkage study (1). Several groups confirmed a significant association between type 2 diabetes and this gene in various populations, with some noteworthy exceptions (2–7). Genome-wide association studies using 300,000–500,000 single nucleotide polymorphisms (SNPs) and high throughput technology overcome the limitation of function-based investigation, and novel susceptibility genes for type 2 diabetes, including zinc transporter (*SLC30A8*), hematopoietically expressed homeobox (*HHEX*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), IGF2 mRNA binding protein 2 (*IGF2BP2*), and CDK5 regulatory subunit associated protein 1-like 1 (*CDKAL1*), have recently been identified. In addition, the fat mass and obesity associated gene (*FTO*) and glucokinase regulatory protein gene (*GCKR*) were associated with body mass index (BMI) and serum triglyceride (TG) level, respectively (8–13). All of these proven genes for type 2 diabetes have been reproducibly associated in multiple studies (14). Meanwhile, exostosin 2 (*EXT2*), *LOC387761* (11), and an intergenic signal (rs9300039) (9) were identified in a single study and have not been replicated. However, most of the populations analyzed were of European ancestry, except in the case of *CDKAL1*, which was replicated in subjects from Hong-Kong. To distinguish variants that are common and reproducible susceptibility genes, it is important to replicate the associations of candidate SNPs with type 2 diabetes in various ethnic groups. In this study we examined the association of recently identified risk SNPs in 10 candidate loci with type 2 diabetes in a relatively large sample set of Japanese subjects.

Subjects and Methods

Subjects

Three sample sets were involved. The Kobe set and the Gunma set subjects were recruited from hospitals in Hyogo and Gunma prefecture, respectively. The Consortium set subjects were recruited from seven districts in Japan by the Study Group of the Millennium Genome Project for Diabetes Mellitus. The inclusion criteria for normal, control subjects of these three sets were as follows: 1) older than 60 yr, 2) glycosylated hemoglobin A_{1c} values less than 5.8%, and 3) no past history of type 2 diabetes. Type 2 diabetes was diagnosed in accordance with World Health Organization criteria. Other forms of diabetes were excluded based on the clinical data. The clinical and laboratory characteristics of the study subjects are shown in supplemental Table 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. Written, informed consent was obtained from all participants. This study was approved by the ethics committee of each participating institute (6).

Genotyping

There were 15 SNPs genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA). These SNPs were selected based on previous reports (8–13) and HapMap linkage disequilibrium (LD) data of Japanese. Departures from Hardy Weinberg Equilibrium were defined as $P < 0.001$ in cases and controls (11). Because SNP rs13266634 in *SLC30A8* was deviated from Hardy Weinberg Equilibrium, SNP rs3802177 in the same gene, which is in strong LD with rs13266634 ($r^2 = 0.96$, HapMap LD data of Japanese), was also examined. The genotyping success rate in the three sample sets was more than 96%. The genotypes determined by TaqMan methods were identical to those determined by direct sequencing for 48 samples. The risk allele of each SNP is shown in supplemental Table 2.

Clinical assessment

The clinical profile of each subject was directly determined at entry. Association studies were performed between the candidate SNPs and BMI, homeostasis model assessment (HOMA) [HOMA of insulin resistance (HOMA-IR) and HOMA of β-cell function (HOMA-β)], or serum TG level. Subjects who had not been treated with insulin were evaluated for HOMA-IR and HOMA-β. Data are expressed as means $\pm$ SD.

Statistical analysis

The differences in SNPs between type 2 diabetic and nondiabetic subjects were compared using χ^2 test and multiple logistic regression analysis under additive, dominant, and recessive models for SNPs. The Cochran-Armitage trend test was also performed with the additive model. There was no heterogeneity among the samples in regard to the recruiting districts. We considered statistical significance at P values less than 0.0033 (0.05/15) in the association study for SNPs after Bonferroni correction. The relation of the variants in these genes with BMI, HOMA-IR, HOMA-β, and TG was assessed by ANOVA for each SNP. The HOMA-IR, HOMA-β, and TG data were log transformed for normality. Statistical analysis was performed with the StatView program (version 5.0-J; SAS Institute Inc., Cary, NC). LD analysis was performed with Haploview (http://www.broad.mit.edu/personal/jcbarret/haploview).

Results

There were 15 SNPs from 10 candidate loci examined for association with type 2 diabetes with a criterion of significance of P value less than $0.05/15 = 0.0033$ after Bonferroni adjustment. Eight SNPs in five loci, *SLC30A8* (rs3802177), *HHEX* (rs1111875, rs7923837), *CDKN2B* (rs10811661), *IGF2BP2* (rs4402960, rs1470579), and *CDKAL1* (rs7754840, rs7756992), were found to be associated with the occurrence of type 2 diabetes (Tables 1 and 2). The P values of association for *CDKN2B* (rs10811661) and *CDKAL1* (rs7754840 and rs7756992) were about 1.9×10^{-6}, 4.5×10^{-7}, and 9.8×10^{-7}, respectively. The next strongest association was found for *IGF2BP2* (rs4402960 and rs1470579) and *HHEX* (rs1111875 and rs7923837) at a P value of 10^{-4}–10^{-5}. SNP rs3802177 of *SLC30A8* showed a nominal association, which disappeared after adjustment for age, sex, and BMI. No association of the other SNPs with type 2 diabetes was detected.

Association studies were also performed between *FTO* and BMI and *GCKR* and serum TG level using the samples with serum data according to previous reports. A nominal association of *GCKR* (rs780094) with serum TG level both in case and control subjects was found in our samples, as previously reported in Caucasians (10). In control subjects the mean values of serum TG were 1.07 ± 0.53, 1.13 ± 0.49, and 1.18 ± 0.55 mmol/liter for CC, CT, and TT genotype, respectively ($P = 0.097$). In cases, these values were 1.32 ± 0.73, 1.43 ± 1.57, and 1.56 ± 1.05 mmol/liter for CC, CT, and TT genotype, respectively ($P = 0.063$). Association of the SNP of *FTO* (rs9939609) with BMI

TABLE 1. Association results between 15 SNPs in 10 candidate loci and type 2 diabetes in Japanese

		Genotype T2DM			Genotype CONT			RAF						95% CI			
Gene	SNP ID	RR	Rr	rr	RR	Rr	rr	T2DM	CONT	*P* value	Armitage trend	*P* value[a]	OR	Upper	Lower	RAF-C	OR -C
SLC30A8	rs13266634	690	806	334	522	725	327	0.60	0.56	3.2×10^{-3}	4.5×10^{-3}	0.17	1.16	1.05	1.27	0.65	1.12
SLC30A8	rs3802177	649	885	306	473	808	291	0.59	0.56	3.2×10^{-3}	3.0×10^{-3}	0.065	1.16	1.05	1.27		
HHEX	rs1111875	212	784	852	132	603	828	0.33	0.28	9.6×10^{-6}	1.4×10^{-5}	8.4×10^{-5}	1.27	1.14	1.40	0.53	1.13
HHEX	rs7923837	98	633	1113	60	467	1049	0.22	0.19	8.8×10^{-5}	1.0×10^{-4}	6.7×10^{-3}	1.27	1.13	1.43	0.62	1.22
LOC387761	rs7480010	1226	556	68	1018	481	67	0.81	0.80	0.33[a]	0.33[a]	0.29	1.06	0.94	1.20		
EXT2	rs3740878	260	842	731	211	738	629	0.37	0.37	0.73[a]	0.74[a]	0.63	1.02	0.92	1.12		
CDKN2B	rs10811661	683	891	283	486	770	326	0.61	0.55	1.7×10^{-6}	1.9×10^{-6}	5.8×10^{-6}	1.27	1.15	1.40	0.83	1.20
CDKN2B	rs564398	1342	482	47	1122	416	41	0.85	0.84	0.67[a]	0.67[a]	0.65	1.03	0.90	1.17	0.56	1.12
GCKR	rs7800944	421	903	534	312	782	492	0.47	0.44	0.029[a]	0.030[a]	0.017	1.11	1.01	1.22		
Inter gene	rs9300039	1068	684	105	903	565	111	0.76	0.75	0.41[a]	0.42[a]	0.15	1.05	0.94	1.17		
IGF2BP2	rs4402960	230	835	787	143	675	759	0.35	0.30	7.9×10^{-5}	8.1×10^{-5}	9.4×10^{-4}	1.23	1.11	1.36	0.29	1.14
IGF2BP2	rs1470579	260	874	738	165	735	694	0.37	0.33	9.0×10^{-4}	8.3×10^{-4}	2.8×10^{-3}	1.18	1.07	1.31	0.30	1.17
CDKAL1	rs7754840	446	881	543	262	781	538	0.47	0.41	3.2×10^{-7}	4.5×10^{-7}	3.5×10^{-7}	1.28	1.17	1.41	0.31	1.12
CDKAL1	rs7756992	537	876	442	330	818	438	0.53	0.47	8.0×10^{-7}	9.8×10^{-7}	3.9×10^{-6}	1.27	1.15	1.40	0.26	1.20
FTO	rs9939609	88	596	1165	63	520	995	0.21	0.20	0.68[a]	0.68[a]	0.74	1.03	0.91	1.15		

ORs, 95% CIs, and *P* values are given for 15 SNPs identified in French, decode, Diabetes Genetics Initiative, Wellcome Trust Case Control Consortium, and Finland-United States Investigation of Noninsulin-Dependent Diabetes Mellitus Genetics studies. SNPs are shown with the risk allele (R) and risk allele frequency (RAF) and the exact count of each genotype in type 2 diabetic (T2DM) patients and controls (CONT). Risk allele-specific ORs and *P* values were calculated using an additive genetic model that in logistic regression is multiplicative on the OR scale. r^2 = 0.83 (rs13266634 and rs3802177), 0.22 (rs1111875 and rs7923837), 0.001 (rs10811661 and rs10811661), 0.87 (rs4402960 and rs1470579), and 0.69 (rs7754840 and rs7756992) in controls of this study. ID, Identification; OR-C, OR in Caucasians; r, nonrisk allele; RAF-C, risk allele frequency in Caucasian controls.

[a] *P* values adjusted for age, sex, and BMI.

TABLE 2. Association results between 15 SNPs in 10 candidate loci and type 2 diabetes in Japanese

		Dominant model					Recessive model				
					95% CI					95% CI	
Gene	SNP ID	P value	P value[a]	OR	Lower	Upper	P value	P value[a]	OR	Lower	Upper
SLC30A8	rs13266634	0.063	0.24	1.17	0.99	1.39	5.8×10^{-3}	0.074	1.22	1.06	1.40
SLC30A8	rs3802177	0.15	0.36	1.14	0.95	1.36	1.3×10^{-3}	0.020	1.27	1.10	1.46
HHEX	rs1111875	6.4×10^{-5}	1.6×10^{-5}	1.32	1.15[a]	1.51	3.5×10^{-3}	0.083	1.40	1.12	1.77
HHEX	rs7923837	1.8×10^{-4}	1.9×10^{-3}	1.31	1.14[a]	1.50	0.036	0.15	1.42	1.02	1.97
LOC387761	rs7480010	0.37	0.16	1.17	0.83[a]	1.65	0.44	0.28	1.06	0.92	1.22
EXT2	rs3740878	0.99	0.66	1.00	0.87[a]	1.15	0.49	0.50	1.07	0.88	1.30
CDKN2B	rs10811661	4.0×10^{-5}	2.3×10^{-5}	1.44	1.21[a]	1.72	1.9×10^{-4}	1.8×10^{-4}	1.31	1.14	1.51
CDKN2B	rs564398	0.88	0.51	1.03	0.68[a]	1.58	0.66	0.65	1.03	0.89	1.20
GCKR	rs7800944	0.14	0.34	1.12	0.96[a]	1.29	0.033	4.2×10^{-3}	1.20	1.01	1.41
Inter gene	rs9300039	0.098	0.20	1.26	0.96[a]	1.66	0.85	0.32	1.01	0.88	1.16
IGF2BP2	rs4402960	9.5×10^{-4}	0.018	1.26	1.10[a]	1.44	1.7×10^{-3}	4.9×10^{-4}	1.42	1.14	1.77
IGF2BP2	rs1470579	0.014	0.063	1.18	1.03[a]	1.36	1.6×10^{-3}	9.2×10^{-4}	1.40	1.13	1.72
CDKAL1	rs7754840	1.6×10^{-3}	1.6×10^{-3}	1.26	1.09[a]	1.46	1.3×10^{-7}	1.5×10^{-7}	1.58	1.33	1.87
CDKAL1	rs7756992	0.01	0.022	1.22	1.05[a]	1.42	4.2×10^{-8}	7.4×10^{-7}	1.55	1.32	1.82
FTO	rs9939609	0.98	0.60	1.00	0.87[a]	1.15	0.28	0.70	1.20	0.86	1.67

ORs, 95% CIs, and *P* values under a dominant or recessive model of each risk allele are given for 15 SNPs identified in French, decode, Diabetes Genetics Initiative, Wellcome Trust Case Control Consortium, and Finland-United States Investigation of Noninsulin-Dependent Diabetes Mellitus Genetics studies. ID, Identification.

[a] *P* values adjusted for age, sex, and BMI.

was found only in control subjects. In addition, subjects with the risk A allele tended to show a larger BMI, as previously reported in Caucasians (13), although it did not reach the level of statistical significance. The mean values of BMI were 22.4 ± 3.2 (TT) and 22.7 ± 3.1 kg/m^2 (AA + AT) for controls ($P = 0.051$). On the other hand, these values were 23.6 ± 3.4 (TT) and 23.8 ± 3.8 kg/m^2 (AA + AT) for cases ($P = 0.306$). Although *HHEX*, *CDKN2B*, *IGF2BP2*, and *CDKAL1* were associated with pancreatic β-cell function in recent reports (15–17), we failed to detect an association with HOMA-β in this study. There was also no evidence of association between HOMA-IR and the risk alleles of these genes.

Discussion

Recent reports have revealed novel type 2 diabetes-susceptibility genes such as *SLC30A8*, *HHEX*, *CDKN2B*, *IGF2BP2*, and *CDKAL1* in the European population (8, 9, 11, 12). In addition, *FTO* and *GCKR* were associated with BMI and serum TG level, respectively (10, 13). In this study we confirmed that all of the proven genes found in Caucasians are replicated in Japanese. The strongest association by *P* value at the 10^{-6}–10^{-7} level was found at *CDKN2B* (rs10811661) and *CDKAL1* (rs7754840, rs7756992), followed by *HHEX* (rs1111875, rs7923837) and *IGF2BP2* (rs4402960, rs1470579). The odds ratio (OR) values of the first three SNPs were 1.27, 1.28, and 1.27, respectively, an even stronger association than that found in the original genome-wide association study in Europeans (10, 14). There were considerable differences in the frequencies of the risk alleles (Table 1), resulting in difficulty of replication due to decreased power of the study in addition to that due to population difference. According to the previous report in Japanese, the SNPs in *HHEX* showed the strongest association with type 2 diabetes, although the frequencies of risk alleles of SNPs in *HHEX* were even lower in the Japanese samples than European populations (15, 18). The previous report showed significant association with both SNPs in *HHEX* but not with the SNPs in *IGF2BP2* (15). This discrepancy cannot be explained by the small sample number because the risk allele frequencies of *IGF2BP2* are higher in Japanese than Europeans, in contrast to those of *HHEX*.

Although the previous study did not detect association of *IGF2BP2* with type 2 diabetes (15), the gene was detected as a diabetes-susceptibility gene in the present study. The absence of significant association in the previous study may be due to the lack of power deriving mainly from the small sample number. Recently, another study in Japanese has reported that rs4402960 in *IGF2BP2* showed the strongest association with type 2 diabetes, using a larger number of samples (19). The present study had 86% power to detect an OR of 1.20 when the frequency of a risk allele was 35% (rs4402960) and the *P* value was less than 0.0033. However, it is important to note that association study is dependent on discrimination of case and control subjects. It also has been reported that lifestyle changes can reduce the risk of type 2 diabetes, even in individuals carrying the type 2 diabetes-susceptibility variant of *TCF7L2* (20).

CDKAL1 and *CDKN2B* showed a nominal association with type 2 diabetes in a previous report in Japanese (15, 19). SNP rs7756992 in *CDKAL1* has been associated with type 2 diabetes in Han Chinese individuals from Hong Kong (12). A strong association between this SNP and type 2 diabetes [OR = 1.27 (95% confidence interval (CI) 1.15–1.40); $P = 9.8 \times 10^{-7}$] was detected in this study. Because type 2 diabetes in Asians is characterized primarily by β-cell dysfunction, these two genes might well be involved in transduction of glucose toxicity or regenerative capacity of pancreatic β-cells and, thus, are possible susceptibility genes for Japanese type 2 diabetes.

We found a nominal association of *GCKR* (rs780094) with the serum TG level both in case and control subjects, as previously reported in Caucasians (10). A nominal association of the

SNP of *FTO* (rs9939609) with BMI was found only in control subjects. In addition, the subjects with risk A allele showed somewhat larger BMI values, as has been reported in Caucasians (13). *FTO* was identified as a type 2 diabetes-susceptibility variant that predisposes to diabetes in the United Kingdom population through its effect on BMI. The lack of association between BMI and the *FTO* SNPs in Japanese could be due to the fact that our samples were from a less obese population.

In conclusion, we were able to replicate a significant association with the largest number of samples so far in Japanese between type 2 diabetes and SNPs in *SLC30A*, *HHEX*, *CDKN2B*, *IGF2BP2*, and *CDKAL1*, which suggests that these variants represent common type 2 diabetes-susceptibility genes in both Japanese and Europeans. Further investigation is required to identify the most likely functional variants.

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References

1. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J, Helgason A, Stefansson H, Emilsson V, Helgadottir A, Styrkarsdottir U, Magnusson KP, Walters GB, Palsdottir E, Jonsdottir T, Gudmundsdottir T, Gylfason A, Saemundsdottir J, Wilensky RL, Reilly MP, Rader DJ, Bagger Y, Christiansen C, Gudnason V, Sigurdsson G, Thorsteinsdottir U, Gulcher JR, Kong A, Stefansson K 2006 Variant of transcription factor 7-like 2 (*TCF7L2*) gene confers risk of type 2 diabetes. Nat Genet 38:320–323
2. Groves CJ, Zeggini E, Minton J, Frayling TM, Weedon MN, Rayner NW, Hitman GA, Walker M, Wiltshire S, Hattersley AT, McCarthy MI 2006 Association analysis of 6,736 U.K. subjects provides replication and confirms *TCF7L2* as a type 2 diabetes susceptibility gene with a substantial effect on individual risk. Diabetes 55:2640–2644
3. Zhang C, Qi L, Hunter DJ, Meigs JB, Manson JE, van Dam RM, Hu FB 2006 Variant of transcription factor 7-like 2 (*TCF7L2*) gene and the risk of type 2 diabetes in large cohorts of U.S. women and men. Diabetes 55:2645–2648
4. Scott LJ, Bonnycastle LL, Willer CJ, Sprau AG, Jackson AU, Narisu N, Duren WL, Chines PS, Stringham HM, Erdos MR, Valle TT, Tuomilehto J, Bergman RN, Mohlke KL, Collins FS, Boehnke M 2006 Association of transcription factor 7-like 2 (*TCF7L2*) variants with type 2 diabetes in a Finnish sample. Diabetes 55:2649–2653
5. Cauchi S, Meyre D, Dina C, Choquet H, Samson C, Gallina S, Balkau B, Charpentier G, Pattou F, Stetsyuk V, Scharfmann R, Staels B, Frühbeck G, Froguel P 2006 Transcription factor *TCF7L2* genetic study in the French population: expression in human beta-cells and adipose tissue and strong association with type 2 diabetes. Diabetes 55:2903–2908
6. Miyake K, Horikawa Y, Hara k, Yasuda k, Osawa H, Furuta H, Hirota Y, Yamagata K, Hinokio Y, Oka Y, Iwasaki N, Iwamoto Y, Yamada Y, Seino Y, Maegawa H, Kashiwagi A, Yamamoto K, Tokunaga K, Takeda J, Makino H, Nanjo K, Kadowaki K, Kasuga M 2008 Association of *TCF7L2* polymorphisms with susceptibility to type 2 diabetes in 4,087 Japanese subjects. J Hum Genet 53:174–180
7. Guo T, Hanson RL, Traurig M, Muller YL, Ma L, Mack J, Kobes S, Knowler WC, Bogardus C, Baier LJ 2007 TCF7L2 is not a major susceptibility gene for type 2 diabetes in Pima Indians: analysis of 3,501 individuals. Diabetes 56: 3082–3088
8. The Wellcome Trust Case Control Consortium 2007 Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447:661–678
9. Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL, Erdos MR, Stringham HM, Chines PS, Jackson AU, Prokunina-Olsson L, Ding CJ, Swift AJ, Narisu N, Hu T, Pruim R, Xiao R, Li XY, Conneely KN, Riebow NL, Sprau AG, Tong M, White PP, Hetrick KN, Barnhart MW, Bark CW, Goldstein JL, Watkins L, Xiang F, Saramies J, Buchanan TA, Watanabe RM, Valle TT, Kinnunen L, Abecasis GR, Pugh EW, Doheny KF, Bergman RN, Tuomilehto J, Collins FS, Boehnke M 2007 A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. Science 316: 1341–1345
10. Diabetes Genetics Initiative of Broad Institute of Harvard, MIT, Lund University, Novartis Institutes of BioMedical Research, Saxena R, Voight BF, Lyssenko V, Burtt NP, de Bakker PI, Chen H, Roix JJ, Kathiresan S, Hirschhorn JN, Daly MJ, Hughes TE, Groop L, Altshuler D, Almgren P, Florez JC, Meyer J, Ardlie K, Bengtsson Boström K, Isomaa B, Lettre G, Lindblad U, Lyon HN, Melander O, Newton-Cheh C, Nilsson P, Orho-Melander M, Råstam L, Speliotes EK, Taskinen MR, Tuomi T, Guiducci C, Berglund A, Carlson J, Gianniny L, Hackett R, Hall L, Holmkvist J, Laurila E, Sjögren M, Sterner M, Surti A, Svensson M, Svensson M, Tewhey R, Blumenstiel B, Parkin M, Defelice M, Barry R, Brodeur W, Camarata J, Chia N, Fava M, Gibbons J, Handsaker B, Healy C, Nguyen K, Gates C, Sougnez C, Gage D, Nizzari M, Gabriel SB, Chirn GW, Ma Q, Parikh H, Richardson D, Ricke D, Purcell S 2007 Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. Science 316: 1331–1336
11. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S, Balkau B, Heude B, Charpentier G, Hudson TJ, Montpetit A, Pshezhetsky AV, Prentki M, Posner BI, Balding DJ, Meyre D, Polychronakos C, Froguel P 2007 A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 445:881–885
12. Steinthorsdottir V, Thorleifsson G, Reynisdottir I, Benediktsson R, Jonsdottir T, Walters GB, Styrkarsdottir U, Gretarsdottir S, Emilsson V, Ghosh S, Baker A, Snorradottir S, Bjarnason H, Ng MC, Hansen T, Bagger Y, Wilensky RL, Reilly MP, Adeyemo A, Chen Y, Zhou J, Gudnason V, Chen G, Huang H, Lashley K, Doumatey A, So WY, Ma RC, Andersen G, Borch-Johnsen K, Jorgensen T, van Vliet-Ostaptchouk JV, Hofker MH, Wijmenga C, Christiansen C, Rader DJ, Rotimi C, Gurney M, Chan JC, Pedersen O, Sigurdsson G, Gulcher JR, Thorsteinsdottir U, Kong A, Stefansson K 2007 A variant in *CDKAL1* influences insulin response and risk of type 2 diabetes. Nat Genet 39:770–775
13. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JR, Elliott KS, Lango H, Rayner NW, Shields B, Harries LW, Barrett JC, Ellard S, Groves CJ, Knight B, Patch AM, Ness AR, Ebrahim S, Lawlor DA, Ring SM, Ben-Shlomo Y, Jarvelin MR, Sovio U, Bennett AJ, Melzer D, Ferrucci L, Loos RJ, Barroso I, Wareham NJ, Karpe F, Owen KR, Cardon LR, Walker M, Hitman GA, Palmer CN, Doney AS, Morris AD, Smith GD, Hattersley AT, McCarthy MI 2007 A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316:889–894
14. Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H, Timpson NJ, Perry JR, Rayner NW, Freathy RM, Barrett JC, Shields B, Morris AP, Ellard S, Groves CJ, Harries LW, Marchini JL, Owen KR, Knight B, Cardon LR, Walker M, Hitman GA, Morris AD, Doney AS, Wellcome Trust Case Control Consortium (WTCCC), McCarthy MI, Hattersley AT 2007 Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. Science [Erratum (2007) 317:1035–1036] 316:1336–1341

15. Horikoshi M, Hara K, Ito C, Shojima N, Nagai R, Ueki K, Froguel P, Kadowaki T 2007 Variations in the *HHEX* gene are associated with increased risk of type 2 diabetes in the Japanese population. Diabetologia 50:2461–2466
16. Pascoe L, Tura A, Patel SK, Ibrahim IM, Ferrannini E, Zeggini E, Weedon MN, Mari A, Hattersley AT, McCarthy MI, Frayling TM, Walker M, RISC Consortium, U.K. Type 2 Diabetes Genetics Consortium 2007 Common variants of the novel type 2 diabetes genes *CDKAL1* and *HHEX/IDE* are associated with decreased pancreatic β-cell function. Diabetes 56:3101–3104
17. Grarup N, Rose CS, Andersson EA, Andersen G, Nielsen AL, Albrechtsen A, Clausen JO, Rasmussen SS, Jørgensen T, Sandbaek A, Lauritzen T, Schmitz O, Hansen T, Pedersen O 2007 Studies of association of variants near the *HHEX, CDKN2A/B*, and *IGF2BP2* genes with type 2 diabetes and impaired insulin release in 10,705 Danish subjects: validation and extension of genome-wide association studies. Diabetes 56:3105–3111
18. Furukawa Y, Shimada T, Furuta H, Matsuno S, Kusuyama A, Doi A, Nishi M, Sasaki H, Sanke T, Nanjo K 2008 Polymorphisms in the *IDE-KIF11-HHEX* gene locus are reproducibly associated with type 2 diabetes in a Japanese population. J Clin Endocrinol Metab 93:310–314
19. Omori S, Tanaka Y, Takahashi A, Hirose H, Kashiwagi A, Kaku K, Kawamori R, Nakamura Y, Maeda S 2008 Association of *CDKAL1, IGF2BP2, CDKN2A/B, HHEX, SLC30A8*, and *KCNJ11* with susceptibility to type 2 diabetes in a Japanese population. Diabetes 57:791–795
20. Florez JC, Jablonski KA, Bayley N, Pollin TI, de Bakker PI, Shuldiner AR, Knowler WC, Nathan DM, Altshuler D, Diabetes Prevention Program Research Group 2006 *TCF7L2* polymorphisms and progression to diabetes in the Diabetes Prevention Program. N Engl J Med 355:241–250

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Effect of fasting on PPARγ and AMPK activity in adipocytes

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ABSTRACT

We investigated the effects of fasting on gene expression and intracellular signals regulating energy metabolism in adipose tissue. Following fasting for 15 h or 39 h, epididymal fat pads were isolated from Wistar rats. PPARγ mRNA levels decreased in the adipose tissues isolated from rats fasted for 39 h, whereas adipocyte lipid-binding protein (aP2) and lipoprotein lipase (LPL) mRNA levels increased. Overnight fasting increased the AMP/ATP ratio and AMP-activated protein kinase (AMPK) in adipose tissue, but not in muscle or liver tissue. In addition, the effect of 5-aminoimidazole-4-carboxyamide-ribonucleoside (AICAR) on PPARγ expression in primary cultured adipocytes was investigated. AICAR reduced PPARγ mRNA levels but increased aP2 and LPL mRNA levels. Thus, fasting-induced AMPK activation may affect on the regulation of gene expression in adipocytes.

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1. Introduction

Obesity is a major health problem commonly associated with insulin resistance, type 2 diabetes, hypertension, dyslipidemia and atherosclerosis. Numerous studies have demonstrated that elevating PPARγ activity with thiazolidinediones improves insulin sensitivity [1]. On the other hand, little is known about the effects of nutritional state, such as a low calorie diet, on the expression and activity of PPARγ in adipose tissue. Herein, we evaluated the expression of PPARγ and related genes in adipose tissue from fasted animals, and assessed the signals regulating glucose and lipid homeostasis in adipose tissue. In particular, we focused on the relationship between fasting and AMP-activated protein kinase (AMPK) in adipocytes.

AMPK is a heterotrimeric enzyme consisting of a catalytic α subunit and regulatory β and γ subunits [2]. AMPK is activated following an increase in the AMP/ATP ratio rather than the intracellular depletion of ATP or accumulation of AMP [3]. Therefore, AMPK is regarded as a fuel gauge whose activation inhibits ATP-consuming pathways. AMPK has emerged as a key regulator of glucose metabolism. Exercise-induced recruitment of Glut4 to the plasma membrane followed by glucose uptake is mediated via AMPK [4]. Thus, considerable work has elucidated the significance of AMPK in glucose and fat homeostasis and its possible usefulness as a therapeutic target in type 2 diabetes. However, little information is available surrounding its roles in adipose tissue, compared with liver, skeletal muscle. Therefore, we propose a possible role for AMPK in adipocytes isolated from fasted animals.

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2. Materials and methods

2.1. Adipocyte preparation

Male Wistar rats weighing 180–200 g were fed ad libitum with a standard diet. To examine the effect of fasting, animals were sacrificed 15 h or 39 h after the removal of food at 18:00. Epididymal fat pads, livers and soleus muscles were collected and homogenized in lysis buffer or Isogen (Nippon Gene) as described previously [5]. For adipocyte primary culture, isolated adipocytes were obtained as described previously [5]. The adipocyte primary culture was maintained in DMEM containing 5% calf serum, with or without 1 mM 5-aminoimidazole-4-carboxyamide-ribonucleoside (AICAR) (Sigma), 3 mM metformin (Sigma), or 10 nM TNFα (Sigma) for 24 h at 37 °C. Cells were then collected in a lysis buffer or Isogen. To assess the role of PPARγ in fasting or the AICAR-induced reaction, a specific PPARγ inhibitor, HX531 was used [6]. Wistar rats were fed with food containing 0.1% HX531 for 1 week, after which time the effects of starvation were evaluated. Isolated adipocytes were incubated with or without 10 μM HX531 and 1 mM AICAR to measure the expression of genes showing a PPAR response element. Furthermore, to evaluate the effect of AMPK inhibitor, on fasting-induced PPARγ and adipocyte lipid-binding protein (aP2) expression, rats were treated intraperitoneally 20 mg/kg compound C, the specific inhibitor of AMPK, prior to fasting [7].

2.2. Immunoblot analysis

Immunoblot was carried out as described previously [5] using anti-AMPK phosphothreonine 172 antibody (Cell Signaling Technology), anti-AMPKα1, α2 antibody (Abcam), anti-phospho-acetyl-CoA carboxylase (ACC) antibody (Upstate Biotechnology), anti-ACC antibody (Upstate Biotechnology).

2.3. AMPK enzymatic activity

AMPK enzymatic activity was measured as described previously [8] using synthetic peptide with sequence HMRSAMSGLHLVKRR as substrate.

2.4. Real-time PCR

The mRNA levels of PPARγ, aP2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured with real-time PCRs as described previously [5]. The mRNA levels of lipoprotein lipase (LPL), leptin and RNA polymerase II (Pol2), an additional housekeeping gene, were measured using real-time PCR. The sense and antisense primers used (GenBank™

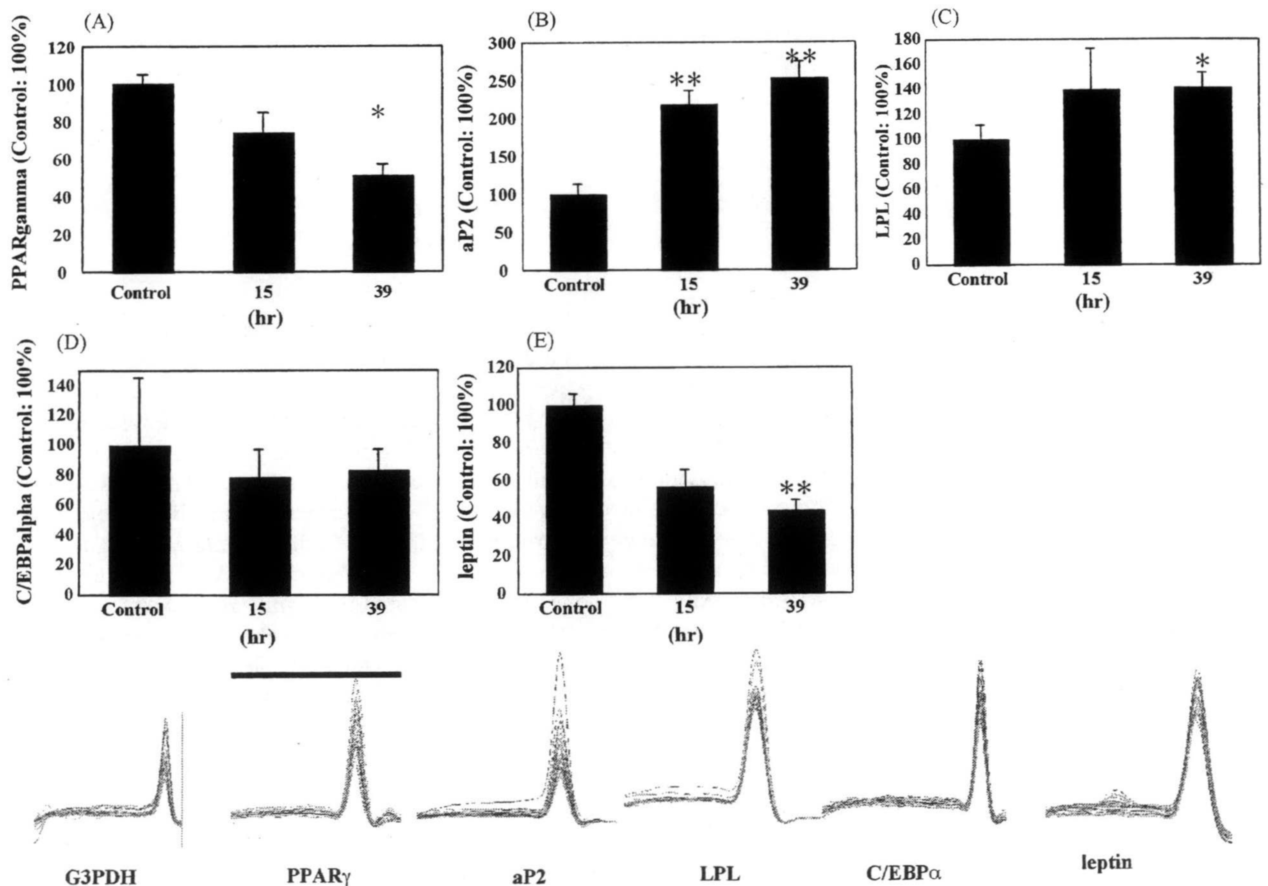

Fig. 1 – Expression of adipocyte specific genes in adipose tissue from starved rats. Wistar rats were fasted for indicated periods, and sacrificed to obtain epididymal fat pads. Total RNA was extracted from each tissue using Isogen, after which real-time PCR was performed. The levels of PPARγ (A), aP2 (B), LPL (C), C/EBPα (D) and leptin (E) mRNA are shown. Values are expressed as means ± S.E. of six determinations. *$p < 0.05$, **$p < 0.005$ by ANOVA.

accession numbers are in parentheses) with the concentrations of Mg^{2+}/dimethylsulfoxide (DMSO) in buffer and annealing temperature were as follows: LPL (NM_012598), nt 956–974 and 1167–1186 with 3 mM Mg^{2+}/2% DMSO at 60 °C; leptin (NM_013076), nt 151–170 and 477–496 with 4 mM Mg^{2+}/2% DMSO at 60 °C; Pol2 (XM_343922), nt 1778–1800 and 1831–1855 with 3 mM Mg^{2+} at 58 °C. Corresponding fragments amplified in the 2 mM Mg^{2+} PCR buffer with Ex Taq DNA polymerase (TaKaRa Co., Osaka, Japan) were gel-purified and quantitated using an Agilent2100 Bioanalyer™ (Agilent Technologies., Palo Alto, CA), yielding each standard to calculate the exact copy number of each mRNA in the samples. The mRNA values calculated as copy numbers in each sample were normalized for a housekeeping gene (GAPDH or Pol2).

2.5. *Nucleotide measurement*

Following treatment with or without overnight fasting, the Wistar rats were sacrificed, and epididymal fat pads, livers and soleus muscles were collected. These samples were washed with PBS before the addition of 5% perchloric acid. Acid-insoluble materials were removed by centrifugation at 10,000 × *g* for 2 min and the pH was adjusted to 7.0 using 3 M K_2CO_3 to 7.0. The amounts of ATP and AMP were measured as described previously [9].

3. Results

Although PPARγ mRNA levels decreased significantly to 51% during the fasting for 39 h (Fig. 1A), mRNA levels of aP2 and LPL, markedly increased to 217% and 141%, respectively, in adipose tissues from rats starved for 15 h (Fig. 1B and C). The activity of AMPK, one of the key enzymes regulating glucose and lipid homeostasis in adipose tissue, was subsequently investigated. Fasting for 15 h resulted in elevated levels of AMPK phosphothreonine 172 and phospho-ACC in adipose tissue (Fig. 2A). In addition, an elevation in the enzymatic activity of AMPK to 210% was observed in fasted adipose tissue (Fig. 2B). In contrast, p-AMPK levels remained unaltered with

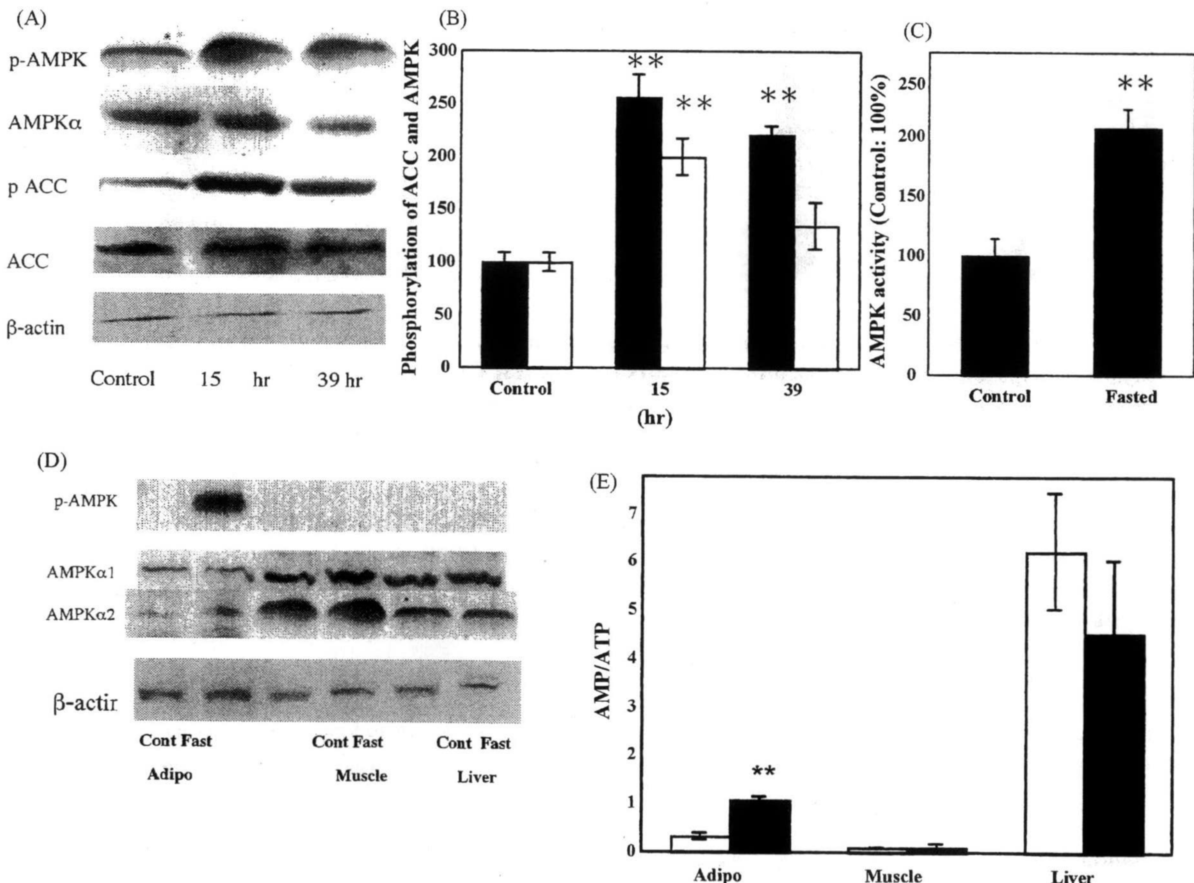

Fig. 2 – Effects of fasting on signals regulating glucose and lipid metabolism. Fasting for 15 h and 39 h increased the AMPK phosphothreonine 172 (p-AMPK) levels and phospho-ACC (p-ACC) levels in adipose tissue. Typical results of Western blotting (A) and quantitated one (filled bars: p-ACC, open bars: p-AMPK, (B) are shown. Values are expressed as means ± S.E. of five determinations. $^{**}p < 0.01$ by ANOVA. Immunoprecipitable AMPK enzymatic activity was assayed by measuring the phosphorylation of synthetic peptide. Values are expressed as means ± S.E. of six determinations. $^{**}p < 0.01$ by ANOVA (C). Although both AMPKα1 and AMPKα2 were expressed in muscle and liver as well as adipose tissue, a fasting-induced increase in p-AMPK levels was detected only in adipose tissue (D). Fasting for 15 h increased AMP/ATP ratio in adipose tissue, but not in soleus muscle or liver. Filled bars represent the results of fasted rats and Open bars represent those of control rats (E). Values are expressed as means ± S.E. of seven determinations. $^{**}p < 0.01$ by ANOVA.

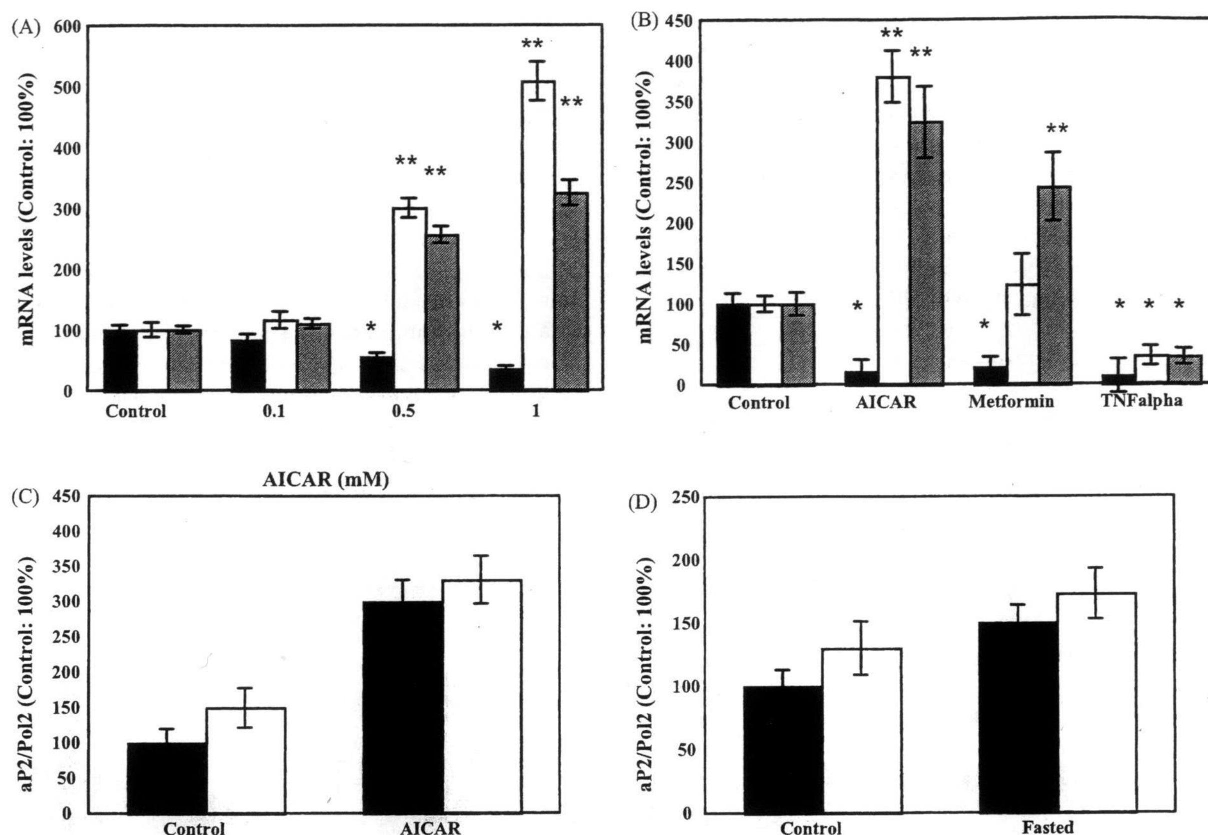

Fig. 3 – Effects of AICAR and other agents on the gene expression in adipocytes. Primary cultured adipocytes were incubated with various concentrations of AICAR (A) or other agents (1 mM AICAR, 3 mM metformin or 10 nM TNFα) (B) for 24 h. Incubation with AICAR reduced the PPARγ (black bars) mRNA levels and elevated aP2 (white bars), LPL (gray bars) and leptin (vertical line bars) mRNA levels. In contrast, treatment with TNFα reduced both PPARγ and aP2 mRNA levels. Values are expressed as means ± S.E. of four determinations. *$p < 0.05$, **$p < 0.01$ by ANOVA. Isolated adipocytes from epididymal fat were pre-incubated with (white bar) or without (black bar) 10 μM HX531 in DMEM for 1 h, then incubated with or without 1 mM AICAR for 24 h (C, left). Wistar rats were bred with (white bar) or without (black bar) 0.1% HX531 containing food. They were fed ad libitum or fasted for 15 h, then epididymal fat tissues were collected to obtain mRNA (D, right). Levels of the aP2 and house keeping gene, Pol2 mRNA in adipocytes was measured using real-time PCR as described in Section 2. Values were expressed as means ± S.E. of three determinations.

fasting in the soleus muscle or liver (Fig. 2C). An increased ratio of AMP/ATP is known to activate AMPK kinase, therefore, we measured nucleotide levels in these tissues. Fasting for 15 h led to a significant increase in the AMP/ATP ratio in adipose tissue, but not in muscle or liver tissue (Fig. 2D). These results strongly suggest that the elevation of the AMP/ATP ratio causes activation of AMPK in adipose tissue.

The possibility that AMPK activation might regulate expression of adipocyte specific genes was then investigated. Incubation with AICAR for 24 h resulted in a dose-dependent decrease in PPARγ mRNA to 31.7% and an increase in aP2, and LPL mRNA to 481% and 302%, respectively, in primary cultured adipocytes (Fig. 3A). Incubation with metformin yielded similar results, whereas incubation with TNFα led to a corresponding reduction in PPARγ and aP2 mRNA (Fig. 3B). Incubation with 1 mM AICAR and 3 mM metformin for 24 h elevated immunoreactive p-AMPK to 260% and 187%, respectively. These results suggested that the activation of AMPK results in elevation of the PPARγ activity *in vivo* as in the case of fasting, however, the expression of PPARγ itself was decreased simultaneously. To assess the role of PPARγ in these apparently conflicting results, the effects of the PPARγ inhibitor, HX531 on fasting and incubation with AICAR were studied. The results showed that HX531 affected neither fasting nor AICAR-induced up-regulation of aP2 (Fig. 3C), but indicated that some other signal may be involved in this process. Similarly, HX531 did not suppress elevation of LPL (data not shown). Administration of metfornin *in vivo* resulted in elevation of aP2 mRNA level, whereas pretreatment with compound C suppressed AMPK activity in fasted rats, which abolished fasting-induced reduction of PPARγ and increase of aP2 mRNA levels (Fig. 4).

4. Discussion

A restricted calorie diet is a common treatment for type 2 diabetes and metabolic syndrome associated with obesity. *In vivo* studies have demonstrated that fasting or a low calorie

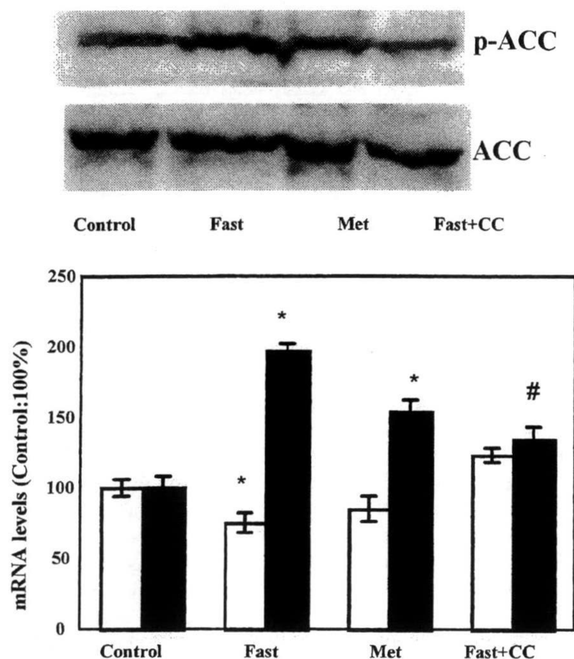

Fig. 4 – Effects of *in vivo* administration of metformin and compound C on the gene expression in adipocytes. To evaluate the pharmacological activation or inhibition of AMPK in vivo, rats were separated into 4 groups ($n = 4$); feeded (Control), fasted (Fast), treated with metformin (Met), treated with compound C (Fast + CC). After oral administration of metfornin (200 mg/(kg day)) for 1 week, epididymal fat was obtained in feeding condition. On the other hand, rats were treated intraperitoneally with compound C (20 mg/kg) before fasting for 15 h. PPARγ (black bar) and aP2 (white bar) mRNA levels were measured with real-time PCR (lower) and phospho-ACC (p-ACC) levels with Western blotting (upper). Values were expressed as means ± S.E. of four determinations. *$p < 0.05$ vs. Control, #$p < 0.05$ vs. Fast.

diet lead to improvement of insulin sensitivity [10]. Moreover, Vidal-Puig et al. demonstrated that a low calorie diet reduced the expression of PPARγ in obese humans [11], although no confirmatory reports have as yet been published. However, little is known about the effects of fasting and feeding on signal transductions regulating energy homeostasis in adipocytes. Herin, we found that fasting for 15 h increased the expression of aP2 and LPL without up-regulation of PPARγ expression. Since LPL is regulated largely post-transcriptionally and post-translationally [12], our results do not mean that fasting increase LPL activity. As expected, leptin mRNA levels were reduced during the fasting.

AMPK was activated during overnight fasting with a concomitant increase in the AMP/ATP ratio observed in adipose tissue only. Recently, Daval et al., reviewed the function of AMPK in adipose tissue [13]. They described that fasting and exercise activate AMPK activity in adipose tissue, however they did not state the tissue specificity and AMP/ATP ratio. We hypothesized that an elevation in AMPK activity might influence gene expression in adipocytes. AICAR, an analog of adenosine, is phosphorylated to form AICAR monophosphate (ZMP). ZMP, like AMP, phosphorylates and activates AMPK [14]. Incubation with AICAR increased the expression of aP2 and LPL, but reduced the expression of PPARγ mRNA significantly. Our results showed that AMPK played a paradoxical role in suppressing the concomitant expression of PPARγ while increasing adipocyte specific genes, which are up-regulated by PPARγ. Currently, we are unable to explain the mechanisms underlying these responses. On the other hand, our results shown in Fig. 3C and D indicated that fasting and AICAR-induced increase in aP2 and LPL mRNA are mediated via other factor than PPARγ. Supraphysiological activation of PPARγ ameliorates insulin sensitivity, whereas moderate reduction of PPARγ activity also improves it [6]. Our results of PPARγ consists with these facts. We simultaneously assessed the effects of other agonists on the expression and *in vivo* activity of PPARγ. Treatment with TNFα reduced the expression of PPARγ, in parallel with aP2 and LPL, probably through the activation of NF-κB. Metformin, which is known to activate AMPK [15], exhibited similar results to those observed with AICAR. These results suggest that activation of AMPK partially mimic the change of gene expression provoked with fasting. Administration of metformoin in vivo yielded the compatible result. Moreover, the result that pharmacological inhibition of AMPK, with compound C prevented fasting-induced effects supported our hypothesis.

Recently, it has been advocated that effects of calorie restriction, including decreased adiposity, repression of PPARγ and increased free fatty acid release, is mediated via Sirt1, an NAD-dependent protein deacetylase [16]. Unfortunately, we have no idea of relation between AMPK and Sirt1 in the role of energy sensing in adipose tissue, however, it is possible that their interaction might regulate total glucose and lipid homeostasis. Calorie restriction, exercise and high plasma adiponectin level activate AMPK in peripheral tissue, which contribute to longevity. On the other hand, SIR2, yeast orthologue of mammalian Sirt1, promotes elongated life span associated with calorie restriction [17]. These facts suggest that both factors might act in common pathway or synergistically. Further study will provide new standpoints for understanding the role of adipocyte and AMPK.

In summary, the present study is the first to evaluate the effects of fasting on the expression and transcriptional activity of PPARγ in adipose tissues. Our results showed that: (1) fasting decreased the expression of PPARγ mRNA and increase the expression of aP2 and LPL mRNA, (2) fasting resulted in an elevated AMP/ATP ratio and activated AMPK activity in adipose tissue, and (3) AMPK may be involved in the fasting-induced gene regulation adipocytes.

Conflict of interest

There are no conflicts of interest.

REFERENCES

[1] J.M. Lehman, L.B. Moore, T.A. Smith-Oliver, W.O. Wilkison, T.M. Willson, S.A. Kliewer, An antidiadetic

thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ), J. Biol. Chem. 270 (1995) 12953–12956.

[2] A. Woods, P.C.F. Cheung, F.C. Smith, M.D. Davison, J. Scott, R.K. Beri, et al., Characterization of AMP-activated protein kinase β and γ subunits. Assembly of the heterotrimeric complex in vitro, J. Biol. Chem. 271 (1994) 10282–10290.

[3] J.M. Corton, J.G. Gillespie, D.G. Hardie, Role of the AMP-activated protein kinase in the cellular stress response, Curr. Biol. 4 (1994) 315–324.

[4] T. Hayashi, M.F. Hirshman, E.J. Kurth, W.W. Winder, L.J. Goodyear, Evidence for 5′AMP activated protein kinase mediation of the effect of muscle contraction, Diabetes 47 (1998) 1369–1373.

[5] K. Kajita, T. Ishizuka, T. Mune, A. Miura, M. Ishizawa, Y. Kanoh, et al., Dehydroepiandrosterone down-regulates the expression of peroxisome proliferator-activated receptor γ in adipocytes, Endocrinology 144 (2003) 253–259.

[6] T. Yamauchi, H. Waki, J. Kamon, K. Murakami, K. Motojima, K. Komeda, et al., Inhibition of RXR and PPARγ ameliorates diet-induced obesity and type 2 diabetes, J. Clin. Invest. 108 (2001) 1001–1013.

[7] L.D. McCullough, Z. Zeng, H. Li, L.E. Landree, J. McFadden, G.V. Ronnett, Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke, J. Biol. Chem. 280 (2005) 20493–20502.

[8] T. Hayashi, M.F. Hirshman, N. Fujii, S.A. Habinowski, L.A. Witters, Metabolic stress and altered glucose transport. Activation of AMP-activated protein kinase as a unifying coupling mechanism, Diabetes 49 (2000) 527–531.

[9] H.U. Bergmeyer, J. Bergmeyer, M. Graßl, Methods of Enzymatic Analysis, 3rd ed., VCH Verlagsgesellschaft mbH, D-6940, Weinheim, Federal Republic of Germany, 1985, pp. 365–370.

[10] Y. Kanoh, G. Bandyopadhyay, M.P. Sajan, M.L. Standaert, R.V. Farese, Rosiglitazone, insulin treatment, and fasting correct defective activation of protein kinase C-ζ/λ by insulin in vastus lateralis muscles and adipocytes of diabetic rats, Endocrinology 142 (2001) 1595–1605.

[11] A.J. Vidal-Puig, R.V. Considine, M.J. Linan, A. Werman, W.J. Pories, J.F. Caro, et al., Peroxisome proliferator-activated receptor gene expression in human tissues; effect of obesity, weight loss, and regulation by insulin and glucocorticoids, J. Clin. Invest. 99 (1997) 2416–2422.

[12] M.H. Doolittle, O. Ben-Zeev, J. Elovson, D. Martin, T.G. Kirchgessner, The response of lipoprotein lipase to feeding and fasting. Evidence for posttranslational regulation, J. Biol. Chem. 265 (1990) 4570–4577.

[13] M. Daval, F. Foufelle, P. Ferre, Functions of AMP-activated protein kinase in adipose tissue, J. Physiol. 574 (2006) 55–62.

[14] J.M. Corton, J.G. Gillespie, S.A. Hawley, D.G. Hardie, 5-Aminoimidazole-4-carboxamide ribonucleoside a specific method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem. 229 (1995) 558–565.

[15] G. Zhou, R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenk-Melody, et al., Role of AMP-activated protein kinase in mechanism of metformin action, J. Clin. Invest. 108 (2001) 1167–1174.

[16] F. Picard, M. Kurtev, N. Chung, A. Topark-Ngarm, T. Senawong, R.M. Oliveira, et al., Sirt1 promotes fat mobilization in white adipocytes by repressing PPARg, Nature 429 (2004) 771–776.

[17] S. Lin, P.A. Defossez, L. Cuarente, Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae, Science 289 (2000) 2126–2128.

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REVIEW

ChREBP: A Glucose-activated Transcription Factor Involved in the Development of Metabolic Syndrome

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Abstract. Excess carbohydrate intake leads to fat accumulation and insulin resistance. Glucose and insulin coordinately regulate *de novo* lipogenesis from glucose in the liver, and insulin activates several transcription factors including SREBP1c and LXR, while those activated by glucose remain unknown. Recently, a carbohydrate response element binding protein (ChREBP), which binds to the carbohydrate response element (ChoRE) in the promoter of rat liver type pyruvate kinase (LPK), has been identified. The target genes of ChREBP are involved in glycolysis, lipogenesis, and gluconeogenesis. Although the regulation of ChREBP remains unknown in detail, the transactivity of ChREBP is partly regulated by a phosphorylation/dephosphorylation mechanism. During fasting, protein kinase A and AMP-activated protein kinase phosphorylate ChREBP and inactivate its transactivity. During feeding, xylulose-5-phosphate in the hexose monophosphate pathway activates protein phosphatase 2A, which dephosphorylates ChREBP and activates its transactivity. ChREBP controls 50% of hepatic lipogenesis by regulating glycolytic and lipogenic gene expression. In ChREBP$^{-/-}$ mice, liver triglyceride content is decreased and liver glycogen content is increased compared to wild-type mice. These results indicate that ChREBP can regulate metabolic gene expression to convert excess carbohydrate into triglyceride rather than glycogen. Furthermore, complete inhibition of ChREBP in ob/ob mice reduces the effects of the metabolic syndrome such as obesity, fatty liver, and glucose intolerance. Thus, further clarification of the physiological role of ChREBP may be useful in developing treatments for the metabolic syndrome.

Key words: ChoRE, ChREBP, L-PK, Metabolic syndrome

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Carbohydrate Response Element Binding Protein (ChREBP)

Increased consumption of high-carbohydrate and high-fat diets (so-called cafeteria diet) is one of the most important risk factors in the development of the metabolic syndrome. Excess carbohydrate is mainly converted to triglyceride in the liver, and excess fat accumulation in the body leads to insulin resistance and metabolic syndrome [1]. When a high-carbohydrate diet is ingested, carbohydrate is converted into triglyceride in the liver by key glycolytic enzymes such as glucokinase and liver-type pyruvate kinase (L-PK) and enzymes of *de novo* lipogenesis such as acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) [2]. Both insulin and glucose are potent factors in inducing the transcription of these key enzyme genes (Fig. 1). Insulin signaling regulates transcription of these glycolytic and lipogenic enzymes by activation of SREBP1c

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Abbreviations: Carbohydrate Response Element Binding Protein (ChREBP), Carbohydrate Response Element (ChoRE), Liver type pyruvate kinase (LPK), Fatty Acid synthase (FAS), Acetyl CoA Carboxylase (ACC), Malic Enzyme (ME), Hexose Monophosphate Shunt (HMP Shunt), Malate-Pyruvate Shunt (MP Shunt), Glucokinase (GCK), glucose 6 phosphatase (G6Pase), Glucose 6 phosphate (G6P), AMP activated protein kinase (AMPK), Xylulose-5-phosphate (Xu-5-P), Protein phosphatase 2A delta (PP2Adelta), cAMP-activated protein kinase (PKA), Phosphoenol Pyruvate (PEP)

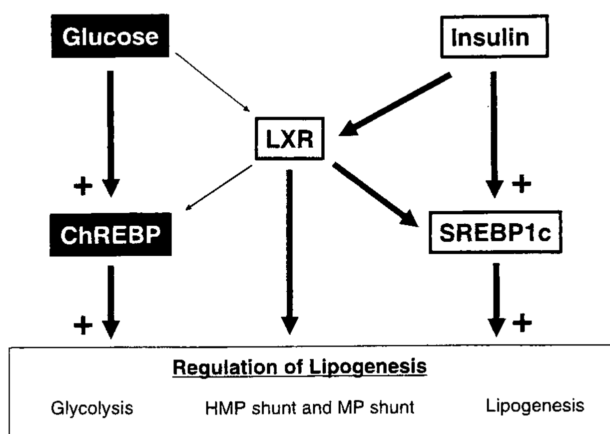

Fig. 1. Glucose and insulin activate transcription factors and regulate *de novo* lipogenesis in liver.
Glucose activates ChREBP and insulin activates SREBP1c and LXR. LXR, liver protein X receptor; SREBP1c, sterol regulatory element binding protein 1c; ChREBP, carbohydrate response element binding protein; HMP, hexose monophosphate; MP, malate-pyruvate shunt.

and liver X receptor (LXR), respectively [3, 4]. In contrast, the mechanism by which glucose signaling activates the expression of these enzymes is poorly understood. For example, transcription of Spot14 (S14) in liver is induced by a carbohydrate diet. Towle *et al.* reported that the carbohydrate response element (ChoRE) of the S14 gene consists of two E-box-like consensus sequences (CAYGNGN5CNCRTG) [5]. Like S14, many glucose response genes (such as LPK, FAS, and ACC) contain a similar ChoRE in their promoter regions [2]. This suggests that a common transcription factor binds to the conserved ChoRE, and many researchers have addressed this problem over a long period [6]. Uyeda *et al.* successfully purified a transcription factor that binds to the rat L-PK ChoRE [7]. This transcription factor is in the same region as the Williams-Beuren syndrome critical region 14 (WBSCR14) protein, which is now renamed the carbohydrate response element binding protein (ChREBP) [7, 8]. Williams-Beuren syndrome is a neurodevelopmental disorder affecting several systems, and is caused by a heterozygous deletion in chromosomal region 7q11.23 in human. WBSCR14/ChREBP is expressed as a 4.2 kb transcript, and the WBSCR14/ChREBP locus encompasses 33 kb of genomic DNA with 17 exons [9]. The distribution of ChREBP mRNA is ubiquitous, but it is most abundant in lipogenic organs such as liver, brown and white adipose tissues, small intestine, kidney, and muscle [7, 10]. ChREBP is a member of the basic helix-loop-helix/leucine zipper (bHLH/ZIP) family of transcription factors with Mr = 94,600, and forms heterodimers with the bHLHZip protein Mlx to bind the ChRoE [8, 11]. Target genes of ChREBP are involved in glycolysis (L-PK), the NADPH supply system (glucose-6-phosphate dehydrogenase, transketolase, malic enzyme, etc.), gluconeogenesis (G6Pase), and lipogenesis (ACC, FAS) [10, 12].

Glucose and insulin signals coordinately regulate lipogenesis

In the fed state, glucose and insulin coordinate hepatic lipogenesis by regulating glycolytic and lipogenic gene expression at the transcriptional level. ChREBP and SREBP1c share lipogenic genes and genes related to the hexose monophosphate (HMP) shunt (Fig. 2) [12, 13]. Some groups have reported that hepatic glucokinase is required for the synergistic effects of ChREBP and SREBP1c on glycolytic and lipogenic gene expression (Fig. 2) [14, 15]. Uyeda *et al.* showed that glucose-activated ChREBP directly binds the ChoRE of the L-PK promoter and activates L-PK gene expression [7]. However, whether SREBP1c physiologically mediates the action of insulin on glucokinase remains controversial. We reconfirmed that the overexpression of dominant active SREBP1c induces glucokinase gene expression in hepatocytes, which we previously confirmed as unpublished data. However, Liang *et al.* reported that the response of glucokinase to high-carbohydrate diet refeeding is still conserved in SREBP1c knockout mice [13]. In addition, Iynedjian *et al.* reported that SREBP1c cannot bind to liver-type glucokinase promoter [16], and Pichard *et al.* reported that SREBP1c knockdown by small interfering RNAs results in impaired induction of the FAS gene in response to glucose and insulin but does not prevent induction of the glucokinase gene [17]. Glucokinase is a key molecule regulating glycolytic flux, and it is important to identify the various transcription factors that mediate the activation of glucokinase gene expression by insulin.

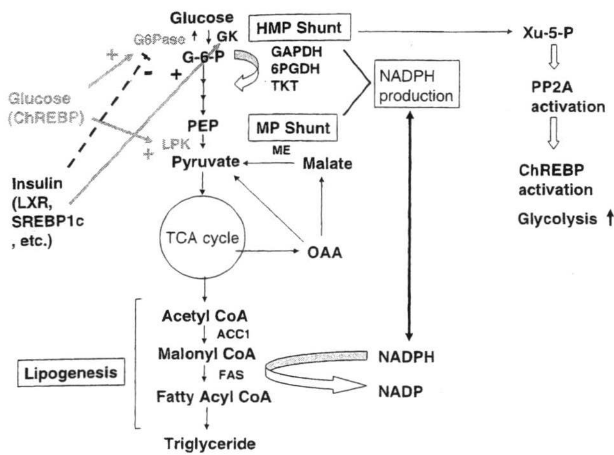

Fig. 2. ChREBP and SREBP-1c regulate different steps in glycolysis and gluconeogenesis.
ChREBP and SREBP share the regulation of lipogenesis and the hexose monophosphate (HMP) and malate-pyruvate (MP) shunts (black and yellow). Glucose (blue) and insulin (red) activate LPK and GK respectively. Glucose also activates G6Pase but insulin inhibits it. G6P, glucose-6-phosphate; GK, glucokinase; G6Pase, glucose-6-phosphatase; PEP, phosphoenol pyruvate; Xu-5-P, xylulose-5-phosphate; ChREBP, carbohydrate response element binding protein; LXR, liver protein X receptor; SREBP1c, sterol regulatory element binding protein 1c; ME, malic enzyme; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; LPK, liver-type pyruvate kinase; OAA, oxaloacetate; Tkt, transketolase; FAS, fatty acid synthase; ACC1, acetyl CoA carboxylase.

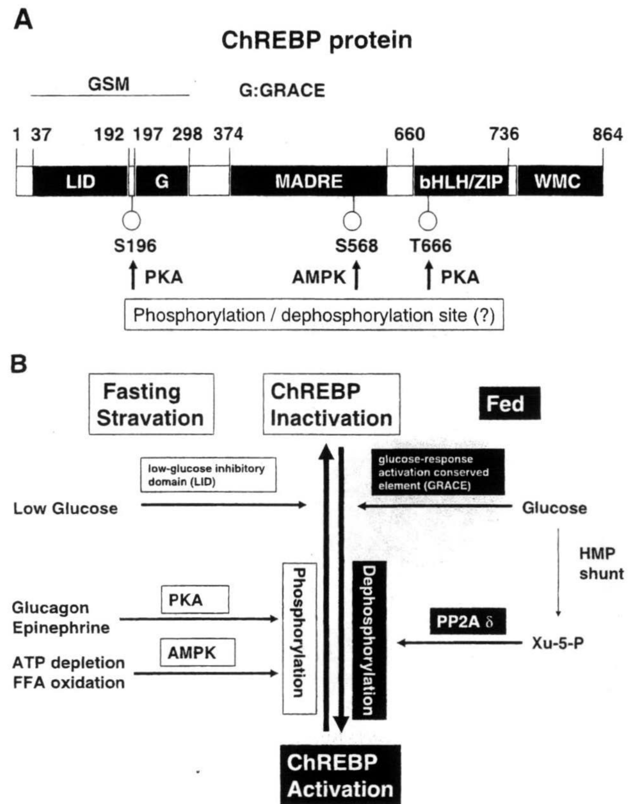

Fig. 3. ChREBP regulation by nutritional state.
(A) ChREBP protein structure. Ser196, Ser568, and Thr666 are putative phosphorylation sites. MADRE, middle activation domain as in RelB; bHLHZip, basic helix-loop-helix leucine zipper domain; WMC, WBSCR14-Mlx C-tail homologous domain; GRACE, glucose response activation conserved element; LID, low-glucose inhibitory domain; GSM, glucose sensing module.
(B) Nutritional conditions determine ChREBP transactivity. PKA, protein kinase A; AMPK, AMP-activated protein kinase; PP2A, protein phosphatase 2A; FFA, free fatty acid; Xu-5-P, xylulose-5-phosphate; HMP, hexose monophosphate; ChREBP, carbohydrate response element binding protein.

Regulation of ChREBP transcriptional activity

Many glycolytic and lipogenic enzymes are induced by high-carbohydrate feeding and suppressed by fasting and starvation [6]. During starvation, hepatic glycolysis and *de novo* lipogenesis are suppressed. In contrast, gluconeogenesis, the beta-oxidation of fatty acyl CoA, and ketogenesis are upregulated. This change from anabolism to catabolism is regulated by stress hormone and AMP accumulation. During starvation, concentrations of plasma glucagon and epinephrine are increased. Glucagon and epinephrine increase the intracellular cAMP concentration and activate cAMP-activated protein kinase (PKA). PKA phosphorylates ChREBP, inactivating it [18]. Phosphorylation of ChREBP at Serine residue 196 (Ser196) inactivates nuclear import, and phosphorylation at Threonine residue 666 (Thr666) prevents DNA binding by ChREBP (Fig. 3A). Similarly, intracellular AMP accumulation inhibits ChREBP transactivity by activating AMP-activated protein kinase (AMPK) and phosphorylating ChREBP (Fig. 3A) [19]. In contrast, glucose activates ChREBP transactivity. Glucose is converted to xylulose-5-phosphate (Xu-5-P) in the hexose monophosphate (HMP) shunt, and Xu-5-P activates protein phosphatase 2A delta (PP2Adelta) and dephosphorylates ChREBP protein (Fig. 3B) [20]. Xu-5-P-mediated PP2A activation also is seen in the activation of 6-phosphofructo-2, 6-kinase/bisphosphatase [21–23]. Xu-5-P is a key molecule in regulat-

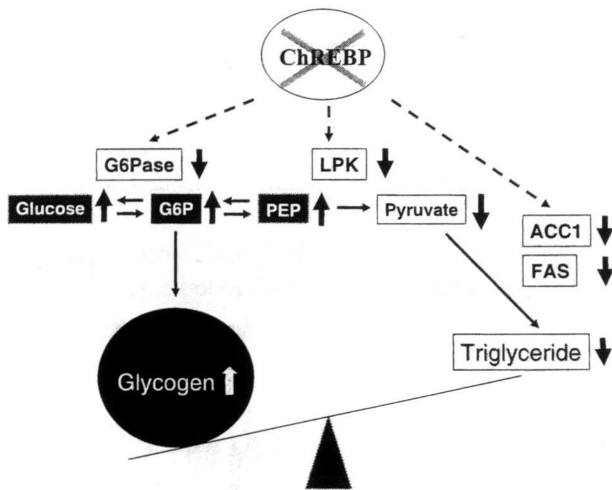

Fig. 4. Deficiency of ChREBP induces glycogen accumulation and decreases triglyceride synthesis in the liver. ChREBP regulates target genes of glycolysis (L-PK), gluconeogenesis (G6Pase) and lipogenesis (FAS, ACC). Excess glycogen accumulation is due to decreased G6Pase and L-PK enzyme activity. Liver triglyceride content is increased by decreased L-PK and lipogenic enzyme activity. G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatse; PEP, phosphoenol pyruvate; ChREBP, carbohydrate response element binding protein; L-PK, liver type pyruvate kinase; GK, glucokinase; OAA, oxaloacetate; Tkt, transketolase.

ing not only transcription but also enzyme activity in glycolysis. Thus, ChREBP, by a phosphorylation/dephosphorylation mechanism, would seem to regulate the expression of glycolytic and lipogenic enzyme genes (L-PK, FAS, ACC, S14, etc.). However, some groups have reported evidence against such a phosphorylation/dephosphorylation mechanism. Despite lacking phosphorylation sites by PKA, a S196A/T666A mutant of ChREBP retains glucose responsiveness and cAMP-dependent inhibition of ACC promoter transactivity [24]. Nevertheless, the ChREBP protein contains a glucose-sensing module that mediates its glucose responsiveness (Fig. 3A and 3B) [25], so it is clear that PKA and AMPK inhibit ChREBP transactivity and that PP2A activates it. However, the mechanism by which glucose activates the transactivity of ChREBP remains unknown (Fig. 4).

At the transcriptional level, ChREBP also is regulated loosely in several conditions. The level of ChREBP mRNA in liver in the fed state is the same as or twice as high as the level during fasting [26]. Repa *et al.* reported that LXR directly regulates ChREBP gene expression at the transcriptional level [27]. The mouse ChREBP gene promoter contains an LXR response element at about 2.4 kbp, and LXR agonists increase hepatic ChREBP mRNA in wild-type mice but not in LXR-α double knockout mice. Moreover, Saez *et al.* reported that LXR is activated by glucose and that high-glucose treatment increased ChREBP mRNA two-fold in HepG2 cells [28]. Insulin also regulates the expression and transactivity of the LXR gene [29]. However, despite the hyperinsulinemia and hyperglycemia seen in ob/ob mice, the level of ChREBP mRNA in liver of ob/ob mice is only twice as high as in liver of wild-type mice [30]. These results suggest that ChREBP transactivity is regulated mainly at the post-transcriptional level rather than at the transcriptional level.

ChREBP knockout mice

To identify the physiological role of ChREBP in hepatic glucose and lipid metabolism, we established ChREBP knockout mice (ChREBP$^{-/-}$) [10]. ChREBP$^{-/-}$ mice are viable and appear to have a normal lifespan. These mice show a phenotype with hepatic lipogenesis from glucose 65% lower than in wild-type mice, and adipose tissue weight correspondingly lower. In addition, the mRNAs of many glycolytic and lipogenic enzymes in liver of ChREBP$^{-/-}$ mice are suppressed [10]. Consistent with the *in vivo* data, our CHIP and EMSA assays show that ChREBP binds directly to ChoREs in the promoters of LPK, ACC, and FAS [31]. These data also indicate that ChREBP directly regulates the expression of glycolytic and lipogenic enzyme genes.

In contrast to decreased lipid content, liver glycogen content is increased and hepatomegaly appears in ChREBP$^{-/-}$ mice [10]. The mechanism by which glycogen content in ChREBP knockout mice is increased is partly understood (Fig. 5). In these mice, most metabolites in the glycolytic pathway, except for pyruvate, are increased. Pyruvate is converted from phosphoenol pyruvate (PEP) by L-PK. In addition, L-PK activity in liver of ChREBP$^{-/-}$ mice was markedly lower and the PEP/pyruvate ratio was higher. G6Pase activity also was decreased in these mice, and the G6P content was increased. Because G6P activates glycogen synthase and stimulates glycogen synthesis in liver, glycogen accumulates in the liver of ChREBP$^{-/-}$

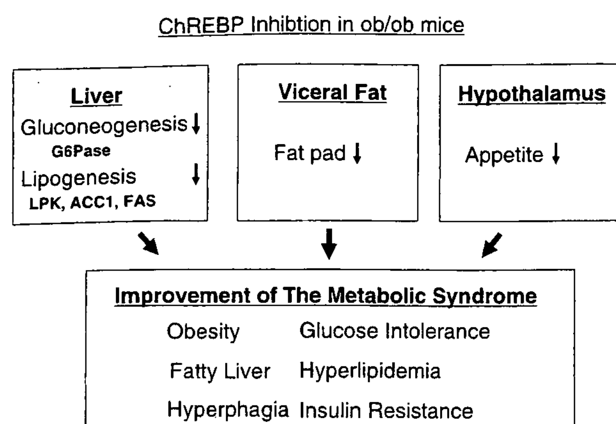

Fig. 5. Deletion of the ChREBP gene improves the metabolic syndrome in ob/ob mice.
In these mice, glycolysis and lipogenesis are increased. Paradoxically, G6Pase activity and gluconeogenesis are increased. When the complete ChREBP gene is deleted, glycolytic genes (LPK) and lipogenic genes (FAS and ACCI) are decreased. Appetite also is decreased in ob/ob ChREBP$^{-/-}$ mice. G6Pase, glucose 6 phosphatase; ChREBP, carbohydrate response element binding protein; LPK, liver-type pyruvate kinase; FAS, fatty acid synthase; ACC1, acetyl CoA carboxylase.

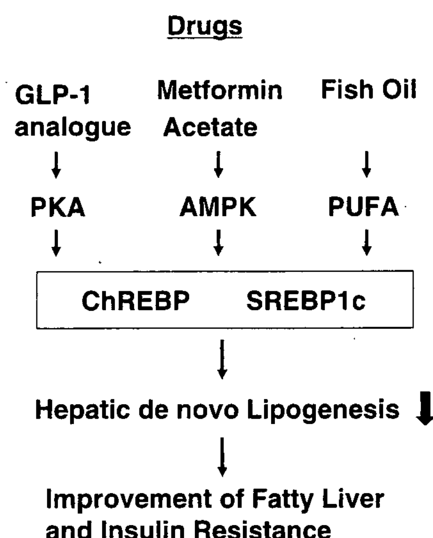

Fig. 6. ChREBP and SREBP1c are potential targets for the treatment of the metabolic syndrome.
PKA, protein kinase A; AMPK, AMP-activated protein kinase; PP2A, protein phosphatase 2A; FFA, free fatty acid; Xu-5-P, xylulose-5-phosphate; HMP, hexose monophosphate; ChREBP, carbohydrate response element binding protein; PUFA, polyunsaturated fatty acids.

mice. These results indicate that ChREBP is a transcription factor that preferentially regulates triglyceride storage (Fig. 5).

Both glucose and insulin are required for full induction of most lipogenic enzyme genes. In addition, the actions of insulin in regard to lipid metabolism are mediated through the transcription factor SREBP1c, and in ChREBP$^{-/-}$ mice, SREBP1 mRNA and protein are unchanged compared with wild-type mice. Furthermore, as with glycolytic genes, SREBP and ChREBP regulate glucokinase and LPK, respectively. These data indicate that ChREBP and SREBP independently regulate *de novo* lipogenesis.

Inhibition of ChREBP as a treatment for metabolic syndrome

Excess fat accumulation in the liver (fatty liver) leads to insulin resistance in the body, and reducing the fat content of the liver readily improves insulin sensitivity [1]. We intercrossed ChREBP$^{-/-}$ mice with ob/ob mice and established ob/ob ChREBP double-cross (ob/ob ChREBP$^{-/-}$) mice [30]. In ob/ob ChREBP$^{-/-}$ mice, complete inhibition of the ChREBP gene throughout the body ameliorated symptoms of the metabolic syndrome such as obesity, insulin resistance, fatty liver, and glucose intolerance (Fig. 6). Consistently, glycolytic and lipogenic gene expression was normalized in ob/ob ChREBP$^{-/-}$ mice. Postic *et al.* reported that delivery of adenovirus-bearing ChREBP short hairpin RNA (Ad-shChREBP) into the liver effectively improved the metabolic syndrome in ob/ob mice [32]. Their data suggest that liver-specific inhibition of ChREBP ameliorates both fatty liver and also glucose intolerance. In contrast, liver-specific inhibition of SREBP1c and PPARγ was found to ameliorate fatty liver but not glucose intolerance [33, 34]. Although the source of the phenotypic differences between these liver-specific SREBP1c or PPARγ knockout mice and Ad-shChREBP mice is not known, ChREBP might regulate G6Pase gene expression at the transcriptional level. G6Pase is a key enzyme in the regulation of gluconeogenesis and glucose output in liver, and decreased G6Pase activity in Ad-shChREBP mice can lower the plasma glucose concentration [32]. Comparison of liver-specific ChREBP knockdown mice with liver-specific SREBP1c or PPARγ knockout mice should provide a clearer understanding of the roles of these substances in the metabolic syndrome.

In addition, food intake in ob/ob ChREBP$^{-/-}$ mice was lower than in ob/ob mice, which was not the case in ob/ob mice infected with Ad-shChREBP. In additon, since ChREBP is also expressed in the brain, ChREBP may regulate appetite control, likely in the hypothalamus (Fig. 6).

Marked hepatomegaly and massive glycogen accumulation are thought to be effects of ChREBP inhibition. Although hepatoma was not visible, excess glycogen accumulation could well finally induce liver fibrosis and carcinogenesis. With the aim of applying these results on ChREBP inhibition to the treatment of metabolic syndrome, we are now identifying ChREBP target genes to suggest new drug therapies based on inhibition of ChREBP transactivity.

Activation of PKA and AMPK has been used in the treatment of obesity-related disorders [35, 36]. Exedine-4 (GLP-1 analogue) increases hepatic cAMP content and ameliorates fatty liver by suppressing *de novo* lipogenesis in ob/ob mice [35]. Metformin also inhibits *de novo* lipogenesis and ameliorates fatty liver by AMPK in genetically obese mice [37]. In addition, acetate can be taken daily in the form of vinegar; when acetate is converted to acetyl CoA, the AMP/ATP ratio is increased and AMPK is activated [38, 39]. Intake of acetate reduces lipogenesis and improves fatty liver in obese mice and rats. In addition, polyunsaturated fatty acids (PUFA) also can be taken daily in the form of fish oil to improve obesity-related disorders [40, 41]. Since these drugs and foods modulate transactivity not only of SREBP but also of ChREBP, they are promising means of mitigating the metabolic syndrome, but the mechanisms by which they act remain unclear.

Role of ChREBP in other tissues

ChREBP is expressed ubiquitously, but mainly in lipogenic organs such as liver, intestine, and white adipose tissues. Interestingly, ChREBP also is expressed in pancreatic islets [42, 43]. In islets, glucose stimulates insulin secretion and is an important signal for cellular events. Using DNA microarrays, many researchers have identified glucose responsive genes in islets that are common to those in liver [44]. In insulin-producing INS-1 cells, overexpression of ChREBP was found to upregulate LPK, FAS, and ACC1 mRNAs, but the insulin response to glucose in these cells was the same as in control cells [42]. In islets of ChREBP$^{-/-}$ mice, glucose-stimulated insulin secretion was the same as in wild-type mice (unpublished data). ChREBP also regulated lipogenic genes in islets, but only overexpression of ChREBP prevented the accumulation of lipid droplets, unlike overexpression of SREBP1c [45]. These findings suggest the action of an insulin signal in addition to ChREBP activation that is important in the induction of lipogenesis.

ChREBP also is abundantly expressed in adipose tissues. During 3T3-L1 preadipocyte adipogenesis, ChREBP is dramatically induced and the expression of its gene in 3T3L1 cells is modulated by various factors including glucose, free fatty acids, insulin, and the antidiabetic agent troglitazone [46]. However, the expression of ChREBP mRNA in adipose tissue *in vivo* is barely responsive to changes in nutrient status. Moreover, ChREBP mRNA is induced in the late stage of adipogenesis and ChREBP has little part in this process. Thus, the physiological role of ChREBP in adipose tissue remains unclear.

Conclusion

The liver is an important organ in the maintenance of glucose homeostasis and energy storage. Excess triglyceride in the liver induces fatty liver and eventually insulin resistance. To prevent the metabolic syndrome, it is important to gain understanding of the mechanism by which certain glucose/insulin-regulated transcription factors coordinate hepatic energy metabolism. Among these transcription factors, glucose-activated transcription factor ChREBP regulates the balance between glycogen and triglyceride storage by coordinately regulating glycolytic and lipogenic gene expression. In genetically obese mice, complete deficiency of ChREBP ameliorates glucose intolerance, fatty liver, and obesity, although hepatomegaly and liver glycogen accumulation develop. Thus, the identification of the roles of ChREBP and its target genes in glucose and lipid metabolism should be useful in developing treatments for the metabolic syndrome.

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References

1. Browning JD, Horton JD (2004) Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 114: 147–152.
2. Towle HC, Kaytor EN, Shih HM (1997) Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annu Rev Nutr* 17: 405–433.
3. Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 109: 1125–1131.
4. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* 14: 2819–2830.
5. Shih HM, Liu Z, Towle HC (1995) Two CACGTG motifs with proper spacing dictate the carbohydrate regulation of hepatic gene transcription. *J Biol Chem* 270: 21991–21997.
6. Girard J, Ferré P, Foufelle F (1997) Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* 17: 325–352.
7. Yamashita H, Takenoshita M, Sakurai M, Bruick RK, Henzel WJ, Shillinglaw W, Arnot D, Uyeda K (2001) A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc Natl Acad Sci USA* 98: 9116–9121.
8. Cairo S, Merla G, Urbinati F, Ballabio A, Reymond A (2001) WBSCR14, a gene mapping to the Williams-Beuren syndrome deleted region, is a new member of the Mlx transcription factor network. *Hum Mol Genet* 10: 617–627.
9. de Luis O, Valero MC, Jurado LA (2000) WBSCR14, a putative transcription factor gene deleted in Williams-Beuren syndrome: complete characterisation of the human gene and the mouse ortholog. *Eur J Hum Genet* 8: 215–222.
10. Iizuka K, Bruick RK, Liang G, Horton JD, Uyeda K (2004) Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc Natl Acad Sci USA* 101: 7281–7286.
11. Stoeckman AK, Ma L, Towle HC (2004) Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J Biol Chem* 27915: 15662–15669.
12. Ma L, Robinson LN, Towle HC (2006) ChREBP*Mlx is the principal mediator of glucose-induced gene expression in the liver. *J Biol Chem* 281: 28721–28730.
13. Liang G, Yang J, Horton JD, Hammer RE, Goldstein JL, Brown MS (2002) Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *J Biol Chem* 277: 9520–9528.
14. Koo SH, Dutcher AK, Towle HC (2001) Glucose and insulin function through two distinct transcription factors to stimulate expression of lipogenic enzyme genes in liver. *J Biol Chem* 276: 9437–9445.
15. Dentin R, Pegorier JP, Benhamed F, Foufelle F, Ferre P, Fauveau V, Magnuson MA, Girard J, Postic C (2004) Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. *J Biol Chem* 279: 20314–20326.
16. Hansmannel F, Mordier S, Iynedjian PB (2006) Insulin induction of glucokinase and fatty acid synthase in hepatocytes: analysis of the roles of sterol-regulatory-element-binding protein-1c and liver X receptor. *Biochem J* 399: 275–283.
17. Gregori C, Guillet-Deniau I, Girard J, Decaux JF, Pichard AL (2005) Insulin regulation of glucokinase gene expression: evidence against a role for sterol regulatory element binding protein 1 in primary hepatocytes. *FEBS Lett* 580: 410–414.
18. Kawaguchi T, Takenoshita M, Kabashima T, Uyeda K (2001) Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. *Proc Natl Acad Sci USA* 98: 13710–13715.
19. Kawaguchi T, Osatomi K, Yamashita H, Kabashima T, Uyeda K (2002) Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. *J Biol Chem* 277: 3829–3835.
20. Kabashima T, Kawaguchi T, Wadzinski BE, Uyeda K (2003) Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose-5-phosphate-activated protein phosphatase in rat liver. *Proc Natl Acad Sci USA* 100: 5107–5112.
21. Nishimura M, Uyeda K (1995) Purification and characterization of a novel xylulose 5-phosphate-activated protein phosphatase catalyzing dephosphorylation of fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase. *J Biol Chem* 270: 26341–26346.
22. Liu YQ, Uyeda K (1996) A mechanism for fatty acid inhibition of glucose utilization in liver. Role of xylulose 5-P. *J Biol Chem* 271: 8824–8830.