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™

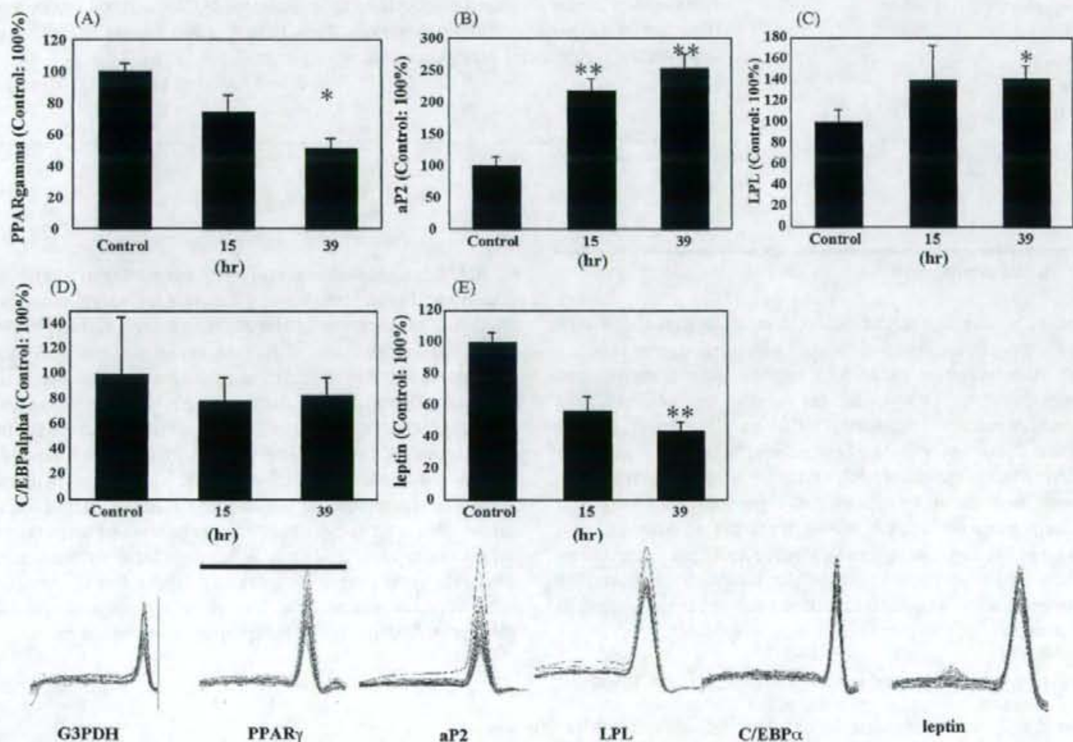

Fig. 1 – Expression of adipocyte specific genes in adipose tissue from starved rats. Wistar rats were fasted for indicated periods, and sacrificed to obtain epididymal fat pads. Total RNA was extracted from each tissue using Isogen, after which real-time PCR was performed. The levels of PPARγ (A), aP2 (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

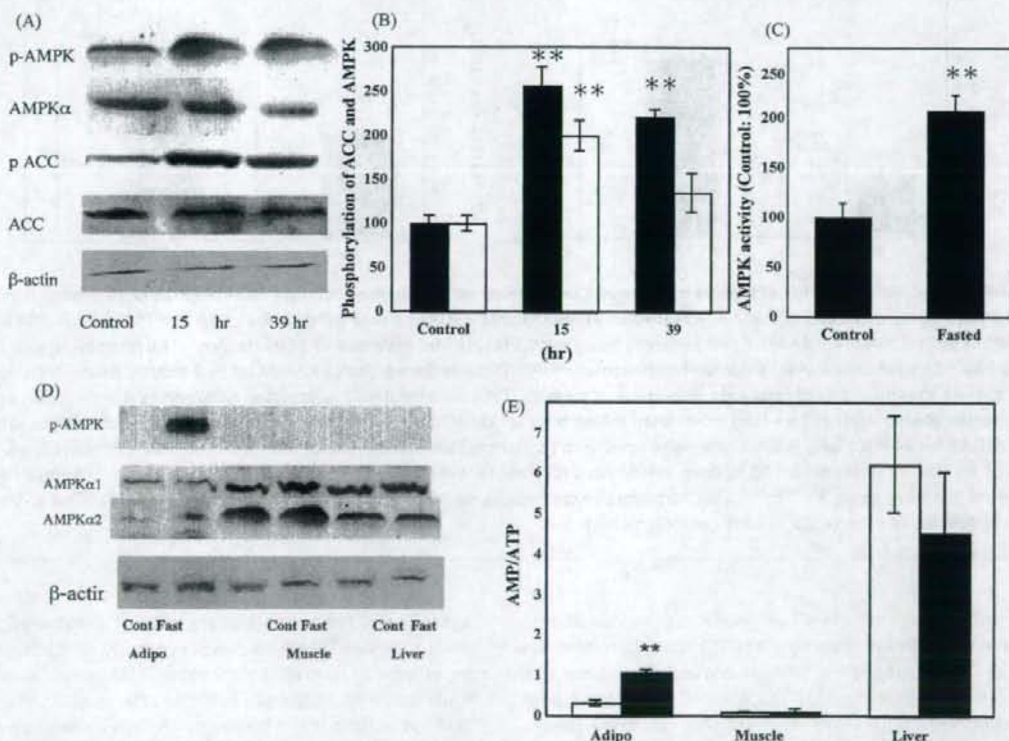

Fig. 2 – Effects of fasting on signals regulating glucose and lipid metabolism. Fasting for 15 h and 39 h increased the AMPK phosphothreonine 172 (p-AMPK) levels and phospho-ACC (p-ACC) levels in adipose tissue. Typical results of Western blotting (A) and quantitated one (filled bars: p-ACC, open bars: 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.

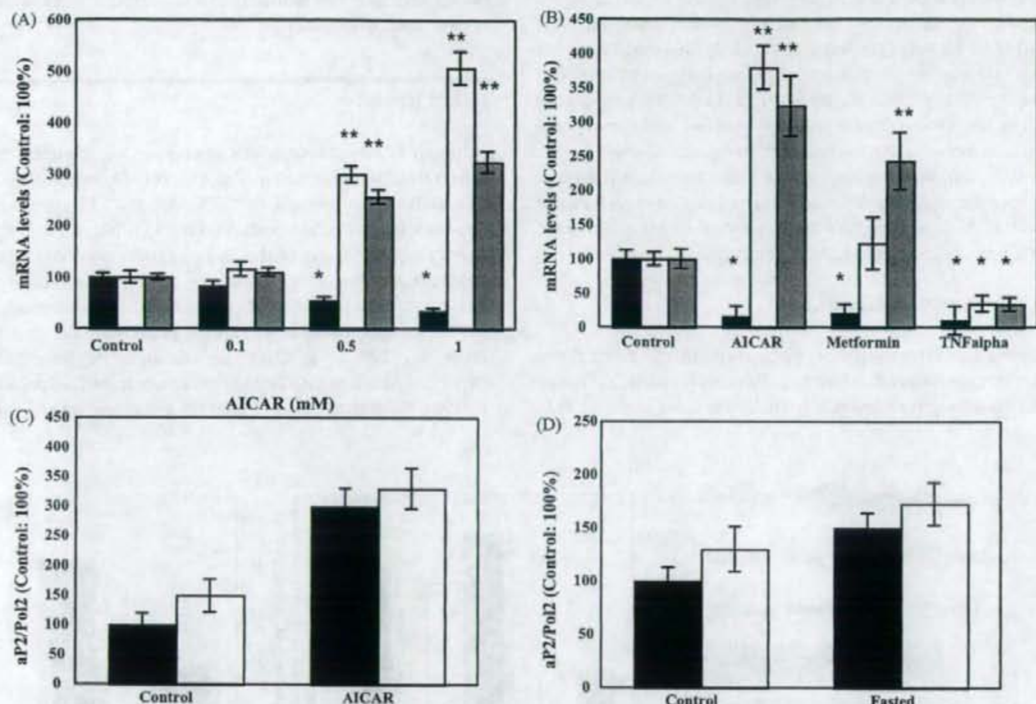

Fig. 3 – Effects of AICAR and other agents on the gene expression in adipocytes. Primary cultured adipocytes were incubated with various concentrations of AICAR (A) or other agents (1 mM AICAR, 3 mM metformin or 10 nM TNFα) (B) for 24 h. Incubation with AICAR reduced the PPARγ (black bars) mRNA levels 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

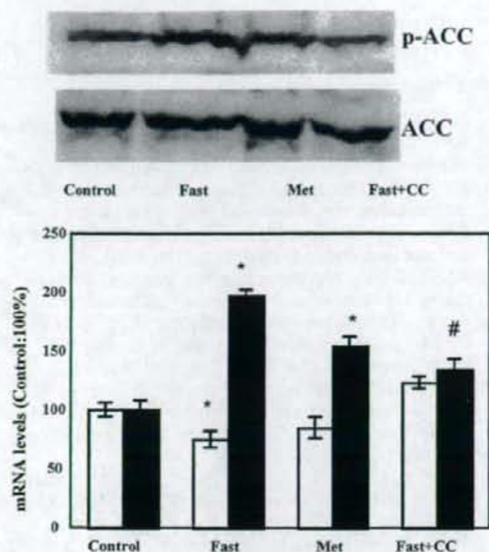

Fig. 4 – Effects of *in vivo* administration of metformin and compound C on the gene expression in adipocytes. To evaluate the pharmacological activation or inhibition of AMPK in vivo, rats were separated into 4 groups ($n = 4$); feeded (Control), fasted (Fast), treated with metformin (Met), treated 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.

Endocrine Journal 2008, **55** (4), 617–624

REVIEW

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

KATSUMI IIZUKA*,*** AND YUKIO HORIKAWA*,**,***

**Laboratory of Medical Genomics, the Institute for Molecular and Cellular Regulation, Gunma University, Maebashi-shi, 371-8512, Japan*

***Department of Diabetes and Endocrinology, Gifu University Graduate School of Medicine, Gifu, 501-1194, Japan*

****Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Kawaguchi, 332-0012, Japan*

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

(*Endocrine Journal* **55**: 617–624, 2008)

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

Received: October 31, 2007
Accepted: October 31, 2007
Correspondence to: Katsumi IIZUKA (kiizuka@showa.gunma-u.ac.jp), Laboratory of Medical Genomics, the Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15, Showa-machi, Maebashi, Gunma, 371-8512, Japan

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)

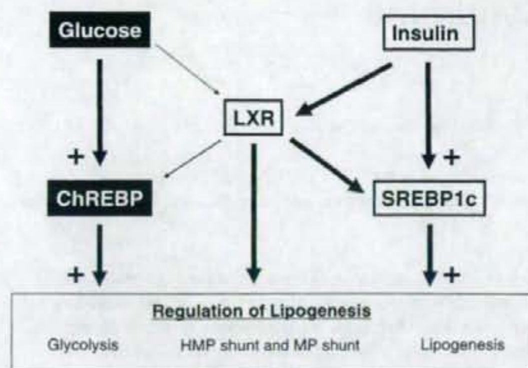

Fig. 1. Glucose and insulin activate transcription factors and regulate *de novo* lipogenesis in liver.
Glucose activates ChREBP and insulin activates SREBP1c and LXR. LXR, liver protein X receptor; SREBP1c, sterol regulatory element binding protein 1c; ChREBP, carbohydrate response element binding protein;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

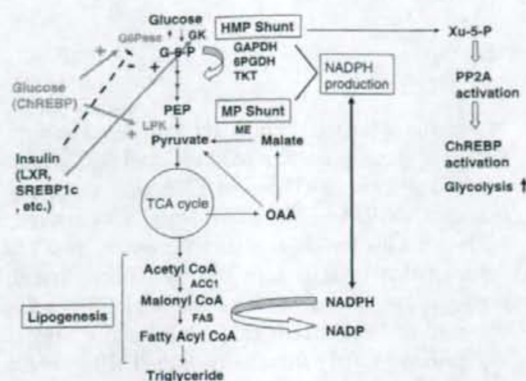

Fig. 2. ChREBP and SREBP-1c regulate different steps in glycolysis and gluconeogenesis.
ChREBP and SREBP share the regulation of lipogenesis and the hexose monophosphate (HMP) and malate-pyruvate (MP) shunts (black and yellow). Glucose (blue) and insulin (red) activate LPK and GK respectively. Glucose 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.

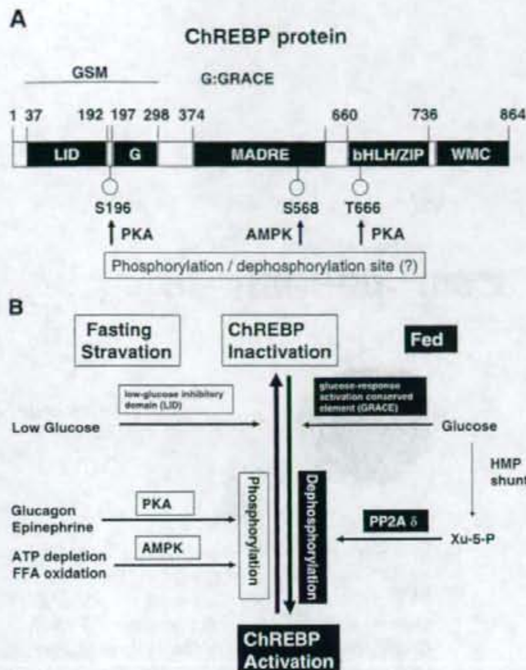

Fig. 3. ChREBP regulation by nutritional state.
(A) ChREBP protein structure. Ser196, Ser568, and Thr666 are putative phosphorylation sites. MADRE, middle activation domain as in RelB; bHLHZip, basic helix-loop-helix leucine zipper domain; WMC, WBSCR14-Mlx C-tail homologous domain; GRACE, glucose response 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-

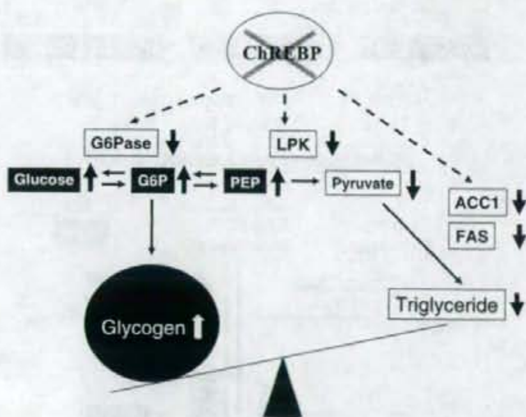

Fig. 4. Deficiency of ChREBP induces glycogen accumulation and decreases triglyceride synthesis in the liver. ChREBP regulates target genes of glycolysis (L-PK), gluconeogenesis (G6Pase) and lipogenesis (FAS, ACC). Excess glycogen accumulation is due to decreased G6Pase and L-PK enzyme activity. Liver 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$^{-/-}$

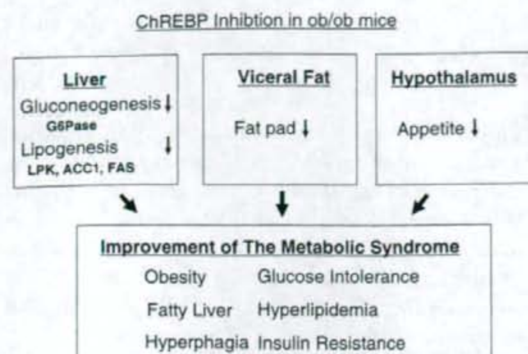

Fig. 5. Deletion of the ChREBP gene improves the metabolic syndrome in ob/ob mice.
In these mice, glycolysis and lipogenesis are increased. Paradoxically, G6Pase activity and gluconeogenesis are increased. When the complete ChREBP gene is deleted, glycolytic genes (LPK) and lipogenic genes (FAS and ACCI) 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.

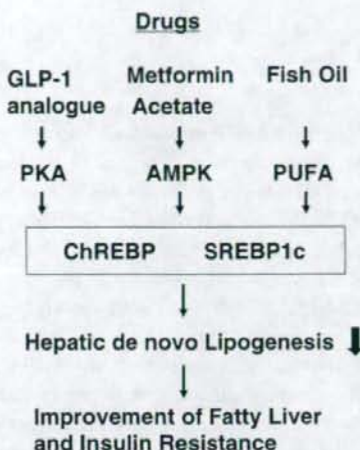

Fig. 6. ChREBP and SREBP1c are potential targets for the treatment of the metabolic syndrome.
PKA, protein kinase A; AMPK, AMP-activated protein kinase; PP2A, protein phosphatase 2A; FFA, free fatty acid; Xu-5-P, xylulose-5-phosphate; HMP, hexose monophosphate; ChREBP, carbohydrate response element binding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

Acknowledgement

We thank Professor Kosaku Uyeda for his guidance and interest. This work was supported in part by a Grant in Aid for scientific research from the Japan So-

ciety for the Promotion of Science (K. Iizuka) and also supported in part by a New Energy and Industrial Technology Development Organization grant to Y. Horikawa.

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.

23. Liu YQ, Uyeda K (1996) A mechanism of regulation of hepatic Fru 2,6-P2 concentration upon refeeding: involvement of xylulose 5-P and cyclic-AMP. *Biochem Biophys Res Commun* 221: 554–558.
24. Tsatsos NG, Towle HC (2006) Glucose activation of ChREBP in hepatocytes occurs via a two-step mechanism. *Biochem Biophys Res Commun* 340: 449–456.
25. Li MV, Chang B, Imamura M, Poungvarin N, Chan L (2006) Glucose-dependent transcriptional regulation by an evolutionarily conserved glucose-sensing module. *Diabetes* 55: 1179–1189.
26. Letexier D, Peroni O, Pinteur C, Beylot M (2005) *In vivo* expression of carbohydrate responsive element binding protein in lean and obese rats. *Diabetes Metab* 31: 558–566.
27. Cha JY, Repa JJ (2007) The liver X receptor (LXR) and hepatic lipogenesis. The carbohydrate-response-element-binding protein is a target gene of LXR. *J Biol Chem* 282: 743–751.
28. Metro N, Mac PA, Vargas L, Godio C, Hampton E, Molteni V, Kreusch A, Saez E (2007) The nuclear receptor LXR is a glucose sensor. *Nature* 445: 219–223.
29. Chen G, Liang G, Ou J, Goldstein JL, Brown MS (2004) Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. *Proc Natl Acad Sci USA* 101: 11245–11250.
30. Iizuka K, Miller B, Uyeda K (2006) Deficiency of carbohydrate-activated transcription factor ChREBP prevents obesity and improves plasma glucose control in leptin-deficient (ob/ob) mice. *Am J Physiol Endocrinol Metab* 291: E358–364.
31. Ishii S, Iizuka K, Miller BC, Uyeda K (2004) Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. *Proc Natl Acad Sci USA* 101: 15597–15602.
32. Dentin R, Benhamed F, Hainault I, Fauveau V, Foufelle F, Dyck JR, Girard J, Postic C (2006) Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes* 55: 2159–2170.
33. Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, Brewer B Jr, Reitman ML, Gonzalez FJ (2003) Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest* 111: 737–747.
34. Yahagi N, Shimano H, Hasty AH, Matsuzaka T, Ide T, Yoshikawa T, Amemiya-Kudo M, Tomita S, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Nagai R, Ishibashi S, Yamada N (2002) Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice. *J Biol Chem* 277: 19353–19357.
35. Ding X, Saxena NK, Lin S, Gupta NA, Anania FA (2006) Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. *Hepatology* 43: 173–181.
36. Long YC, Zierath JR (2006) AMP-activated protein kinase signaling in metabolic regulation. *J Clin Invest* 116: 1776–1783.
37. Lin HZ, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, Diehl AM (2000) Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med* 6: 998–1003.
38. Sakakibara S, Yamauchi T, Oshima Y, Tsukamoto Y, Kadowaki T (2006) Acetic acid activates hepatic AMPK and reduces hyperglycemia in diabetic KK-A(y) mice. *Biochem Biophys Res Commun* 344: 597–604.
39. Yamashita H, Fujisawa K, Ito E, Idei S, Kawaguchi N, Kimoto M, Hiemori M, Tsuji H (2007) Improvement of obesity and glucose tolerance by acetate in Type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Biosci Biotechnol Biochem* 71: 1236–1243.
40. Sekiya M, Yahagi N, Matsuzaka T, Najima Y, Nakakuki M, Nagai R, Ishibashi S, Osuga J, Yamada N, Shimano H (2003) Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* 38: 1529–1539.
41. Dentin R, Benhamed F, Pégorier JP, Foufelle F, Viollet B, Vaulont S, Girard J, Postic C (2005) Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation. *J Clin Invest* 115: 2843–2854.
42. Wang H, Wollheim CB (2002) ChREBP rather than USF2 regulates glucose stimulation of endogenous L-pyruvate kinase expression in insulin-secreting cells. *J Biol Chem* 277: 32746–32752.
43. da Silva Xavier G, Rutter GA, Diraison F, Andreolas C, Leclerc I (2006) ChREBP binding to fatty acid synthase and L-type pyruvate kinase genes is stimulated by glucose in pancreatic beta-cells. *J Lipid Res* 47: 2482–2491.
44. Webb GC, Akbar MS, Zhao C, Steiner DF (2000) Expression profiling of pancreatic beta cells: glucose regulation of secretory and metabolic pathway genes. *Proc Natl Acad Sci USA* 97: 5773–5778.
45. Wang H, Kouri G, Wollheim CB (2005) ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. *J Cell Sci* 118: 3905–3915.
46. He Z, Jiang T, Wang Z, Levi M, Li J (2004) Modulation of carbohydrate response element-binding protein gene expression in 3T3-L1 adipocytes and rat adipose tissue. *Am J Physiol Endocrinol Metab* 287: E424–430.

MUTATION IN BRIEF

Mutations in the Small Heterodimer Partner Gene Increase Morbidity Risk in Japanese Type 2 Diabetes Patients

Mayumi Enya[1§], Yukio Horikawa[1,2§*], Eiji Kuroda[1], Kayoko Yonemaru[1], Naoko Tonooka[2], Hideaki Tomura[2], Naohisa Oda[3], Norihide Yokoi[4], Kazuya Yamagata[5†], Nobuyuki Shihara[2], Katsumi Iizuka[2], Toshiji Saibara[6], Susumu Seino[4], and Jun Takeda[1]

From the [1]Department of Diabetes and Endocrinology Division of Molecule and Structure Gifu University School of Medicine, Gifu; the [2]Laboratory of Medical Genomics, Biosignal Genome Resource Center, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi; the [3]Department of Internal Medicine, Fujita Health University School of Medicine, Aichi; the [4]Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe; the [5]Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Osaka, the [6]Department of Gastroenterology and Hepatology, Kochi Medical School, Nankoku, Japan

† present affiliation: *Department of Medical Biochemistry, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University*

§ These authors equally contributed to this work

*Correspondence to; Yukio Horikawa, MD, PhD

Department of Diabetes and Endocrinology, Division of Molecule and Structure, Gifu University School of Medicine, Gifu

TEL: +81-58-230-6564, FAX: +81-58-230-6376, E-mail: yhorikaw@gifu-u.ac.jp

Contract grant sponsor: This work was supported by a Health and Labor Science Research Grant for research on Human Genome and Tissue Engineering from the Japanese Ministry of Health, Labor and Welfare, a Grant-in-Aid for Scientific Research from the Japanese Ministry of Science, Education, Sports, Culture and Technology, and a New Energy and Industrial Technology Development Organization Grant.

Communicated by Michael Dean

Mutations in the small heterodimer partner gene (NR0B2; alias SHP) are associated with high birth weight and mild obesity in Japanese children. *SHP* mutations may also be associated with later obesity and insulin resistance syndrome that induces diabetes. To investigate this possibility, the prevalence of *SHP* mutations in Japanese with and without type 2 diabetes mellitus and the functional properties of the mutant proteins were evaluated. Direct sequencing of two exons and flanking sequences of *SHP* in 805 diabetic patients and 752 non-diabetic controls identified 15 different mutations in 44 subjects, including 6 novel mutations. Functional analyses of the mutant proteins revealed significantly reduced activity of nine of the mutations. Mutations with reduced activity were found in 19 patients (2.4%) in the diabetic group and in 6 subjects (0.8%) in the control group. The frequency difference between DM and control subjects adjusted for sex and age was statistically significant (P=0.029, odds ratio 2.67, 95% CI 1.05 – 6.81, 1-β=0.91). We conclude that *SHP* mutations associated with mild obesity in childhood increase susceptibility to type 2 diabetes in later life in Japanese. © 2008 Wiley-Liss, Inc.

KEY WORDS: SHP, type 2 diabetes, obesity, fatty liver, NASH

Received 16 April 2008; accepted revised manuscript 20 June 2008.

© 2008 WILEY-LISS, INC.

DOI: 10.1002/humu.20865

INTRODUCTION

Type 2 diabetes mellitus is characterized by defects of insulin secretion in pancreatic b-cells and insulin action in peripheral tissues. Failure of pancreatic β-cells to compensate for insulin resistance by increasing insulin secretion is thought to underlie the development of type 2 diabetes (Reaven, 1988, Polonsky, 2000).

We have previously shown that mutations in the gene encoding small heterodimer partner (*NR0B2*, alias *SHP*; MIM# 604630), an orphan nuclear receptor that interacts with a number of other receptors (Seol et al., 1996, Masuda et al., 1997, Seol et al., 1998, Johansson et al., 1999), are associated with high birth weight and mild obesity in Japanese children, although the molecular mechanisms by which the *SHP* mutations cause these disorders are unknown (Nishigori et al., 2001).

Nuclear receptors such as SHP and peroxisome proliferator-activated receptor (PPAR) α that regulate lipid metabolism in liver are potential contributors to fatty liver. It should be noted that the storage of lipids in liver can trigger inter-organ crosstalk systems that affect insulin sensitivity in muscle. Farnesoid X receptor (FXR)-null mice, with reduced levels of SHP, develop severe fatty liver and elevated circulating FFAs, which is associated with elevated serum glucose and impaired glucose and insulin tolerance resulting from attenuated inhibition of hepatic glucose production by insulin and reduced peripheral glucose disposal (Ma et al., 2006). Some patients with *SHP* mutations exhibit liver dysfunction due to fatty liver (Nishigori et al., 2001). Accordingly, mutations in *SHP* may be associated with insulin resistance due to both later obesity and also to fatty liver in Japanese subjects.

Nonalcoholic fatty liver disease (NAFLD) is a polygenic disease caused by a combination of environmental and genetic factors. Potential candidate genes contributing to NAFLD, a condition comprising a spectrum of pathological liver conditions ranging from steatosis alone to non-alcoholic steatohepatitis (NASH), include those involved in fat deposition, insulin sensitivity, and hepatic lipid oxidation, synthesis, storage, and export. NASH is believed to be a hepatic expression of metabolic syndrome (Ono and Saibara, 2006). In this regard, genetic abnormalities manifested in obesity and fatty liver might well act in concert to induce diabetes.

To evaluate the influence of *SHP* mutations on risk of later development of type 2 diabetes, we examined the frequencies of these mutations in Japanese subjects with and without type 2 diabetes mellitus as well as in patients with NASH.

MATERIALS AND METHODS

Patient populations

The ADA definitions of type 2 diabetes were used. Obesity is defined in these studies as BMI of >25 kg/m^2, in accord with the criteria of the Japan Society for the Study of Obesity (Japanese Society for the Study of Obesity, 2000) and the report by WHO (Western Pacific Region) and IASO/IOTF (International Association for the Study of Obesity/International Obesity Task Force) (WHO and IASO/IOTF, 2000). We evaluated the prevalence of SHP mutations in 805 Japanese patients with type 2 diabetes (male/female, 432/373: age, 60.3±11.8 yr.; BMI, 24.1± 4.0 kg/m^2) and 752 non-diabetic controls (male/female, 418/334; age, 59.7±13.3 yr.; BMI, 22.9±2.9 kg/m^2). Informed consent was obtained from all of the diabetic subjects and volunteer controls. NASH patients with nonalcoholic fatty liver disease underwent liver biopsy after signed informed consent and thorough clinical evaluation. Liver biopsy was analyzed by a pathologist (H.E.) and the diagnosis of NASH was based on Brunt's criteria (Brunt et al., 1999). Laboratory blood tests and BMI were analyzed in 93 biopsy-proved NASH patients (48 males and 45 females, age: 29.2 ± 5.4 years old, BMI: 29.2 ± 5.4 kg/m2, ALT: 102.6 ± 66.6 IU/L, T Chol: 5.25 ± 1.05 mmol/L, TG: 1.74 ± 0.88 mmol/L, HDL-C: 1.24 ± 0.35 mmol/L, HbA1c: 5.6 ± 1.0 %, FPG: 6.03 ± 1.79 mmol/L).

Mutation analysis

The two exons and flanking regions of the SHP gene were screened for mutations by direct DNA sequencing of the amplified polymerase chain reaction (PCR) products, using specific primer pairs and an ABI PRISM BigDye Terminator Cycle Sequencing FS ready Reaction Kit (Applied Biosystems, Foster City, CA). Primer pairs and PCR conditions used for screening of the SHP gene are as follows. Exon1: 5'-CATGACTTCTGGAGTCAAGG-3' and 5'-GTCCCTTTCAGGCAGGCATA-3',

5'-CATCCTTCTGGCAGCTGCCT-3' and 5'-TTAGAAGCTACCTTCCCTGGCT

GG-3' Exon2: 5'-CAGATCTTGGGCCAGTCTTG-3' and 5'-CTCCAGGAGCATTG GGTCAC-3'. Genomic DNA extracted from diabetic and control subjects was initially denatured at 95° C for 1 min, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 60° C or 62° C for 30 sec, extension at 72° C for 30 sec, and a final extension step of 7 min. The sequencing reactions were analyzed by automatic DNA sequencers (Applied Biosystems models 3100 and 3700).

Mutation Nomenclature

The cDNA NM_021969.1 and protein NP_068804.1 sequences were used for mutation nomenclature, with DNA +1 corresponding to the A of the ATG translation initiation codon. Descriptions of all sequence variants were checked using the Mutalyzer program (http://www.LOVD.nl/mutalyzer/).

Functional analysis of SHP mutant proteins

Analysis of the functional properties of mutant and wild-type proteins was performed as described previously (Nishigori et al., 2001). Briefly, the *SHP* mutations newly identified in this study were generated by PCR-based site-directed mutagenesis and cloned in the expression pCMV-6b vector. The sequences for wild-type and mutant SHP proteins, and HNF-4α were cloned in pCMV-6b and pcDNA3.1 (Invitrogen, Groningen, The Netherlands), respectively. For luciferase reporter assays, the promoter region of the human HNF-1α gene was inserted into the pGL3-Basic Reporter vector (Promega, Madison, WI).

HepG2 cells ($1\mathrm{x}10^5$) were grown in 6-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum. The cells were transfected with ExGen 500 solution (6.6 ml) (Fermentas, Ontario, Canada), 333 ng of HNF-1α-promoter/reporter construct, 100 ng of HNF-4α-expression plasmid, 0-125 ng of test DNA, and 17 ng of pRL (Renilla luciferase)-TK. Luciferase reporter activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Renilla luciferase activity was used to normalize transfection efficiencies among experiments.

Statistical analyses

Statistical difference in frequencies of *SHP* mutations between the diabetic and control groups was analyzed by logistic regression analysis, using a package of STATVIEW 5.0 (SAS Institute Inc., Cary, NC). Data obtained by luciferase reporter assay were analyzed by the Student's *t*-test.

RESULTS

Eight hundred five Japanese patients with adult-onset type 2 diabetes (T2DM), 752 non-diabetic controls, and 93 patients with NASH were examined. Screening of the SHP gene (*NR0B2*) by direct sequencing resulted in the identification of fifteen different mutations (c.100C>T [p.Arg34X], c.112C>T [p.Arg38Cys], c.134G>C [p.Arg45Pro], c.157_166del [p.His53AlafsX50], c.160C>T [p.Arg54Cys], c.169C>T [p.Arg57Trp], c.292_300delinsAC [p.Leu98ThyfsX6], c.314T>G [p.Val105Gly], c.512G>C [p.Gly171Ala], c.532G>A [p.Asp178Asn], c.566G>A [p.Gly189Glu], c.583G>T [p.Ala195Ser], c.618G>A [p.Trp206X], c.637C>T [p.Arg213Cys], and c.647G>A [p.Arg216His]) including six novel mutations in type 2 diabetic patients (Table 1),

eight of which were previously identified in obese children (Nishigori et al., 2001) and one of which, p.Gly171Ala, was reported as a polymorphism in a study of Caucasians (Hung et al., 2003, Echwald et al., 2004, Mitchell et al., 2003). In NASH patients, only one mutation, p.Arg45Pro, was identified. We could not find any variants in flanking sequences.

Table 1: Mutations identified in the human SHP gene (*NR0B2*).

Exon	Codon	Nucleotide change	Designation	Patients (n=805)	Controls (n=752)
Mutations with reduced activity					
1	34	c.100C>T	p.Arg34X [a)b)c)d)e)]	2	0
1	53	c.157_166del	p.His53AlafsX50 [a)b)c)e)]	2	0
1	54	c.160C>T	p.Arg54Cys*	0	1
1	57	c.169C>T	p.Arg57Trp [a)]	1	0
1	98	c.292_300 delinsAC	p.Leu98ThyfsX6 [a)c)e)]	6	1
1	105	c.314T>G	p.Val105Gly*	1	0
2	189	c.566G>A	p.Gly189Glu [a)]	3	0
2	195	c.583G>T	p.Ala195Ser [a)c)d)e)]	1	3
2	206	c.618G>A	p.Trp206X*	2	1
2	213	c.637C>T	p.Arg213Cys [a)b)c)e)]	1	0
			sum	19 (2.4%)	6 (0.8%)
Mutations with normal activity					
1	38	c.112C>T	p.Arg38Cys*	1	0
1	45	c.134G>C	p.Arg45Pro*	1	0
1	171	c.512G>C	p.Gly171Ala	0	1
1-2	178	c.532G>A	p.Asp178Asn*	1	0
2	216	c.647G>A	p.Arg216His	6	9
			sum	9 (1.1%)	10 (1.3%)

* indicates six novel variants identified in the present study.
To determine if the mutations alter the function of the SHP protein, the effect of the wild-type and mutant proteins on HNF-4α-mediated transactivation of HNF-1α gene transcription in HepG2 cells was examined by luciferase reporter assay (Fig. 1 and Nishigori et al., 2001). a) early-onset obesity, b) high birth weight, c) diabetes, d) fatty liver, e) decreased insulin sensitivity (Nishigori et al., 2001) Mutations were numbered according to GenBank NM_021969.1 and NP_068804.1. Nucleotide +1 is A of the ATG initiation codon.

Functional analyses of the novel mutant proteins showed significantly reduced activity of transcriptional regulation of HNF-4α, except in the case of p.Arg38Cys, p.Arg45Pro, and p.Asp178Asn. The results of functional analyses of the mutations newly identified in this study are shown in Fig. 1. The mutations with reduced activity were found in nineteen subjects (2.4%) in the diabetic group, six (0.8%) in the control group, and none in the NASH group (Table 1). The frequency difference between DM and control groups was statistically significant by logistic regression analysis considering gender and age (P=0.029; 1-β =0.91) with odds ratio of 2.67 [95% CI, 1.05-6.81]. This frequency difference between DM and control groups came to be not statistically significant by logistic regression analysis when considering gender, age, and BMI (P=0.078), with odds ratio of 2.26 [95% CI, 0.87-5.86]. Furthermore, subjects with mutations of reduced activity showed significantly higher BMI than subjects without the mutations (25.6 ± 4.6 vs 23.5 ± 3.6, P=0.0039 in combined subjects, and 24.0 ± 4.0 vs. 26.5 ± 5.0, P=0.012 in diabetic patients). In control subjects, those with mutations of reduced activity showed similar BMI to those without mutations (22.9 ± 2.9 vs. 23.1 ± 2.7, P=0.87).

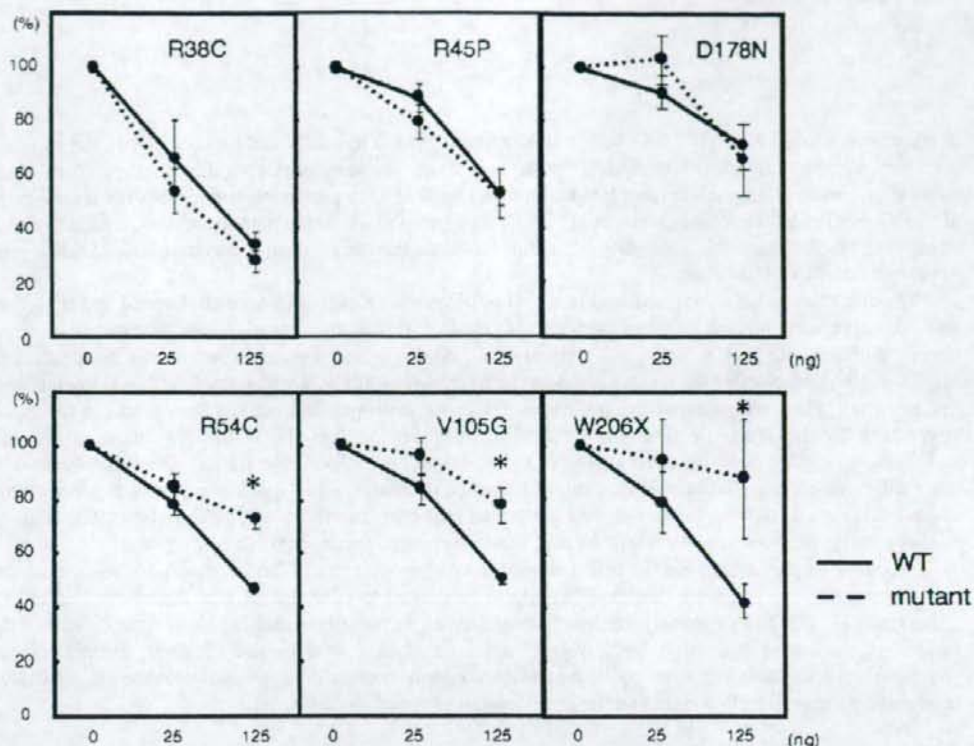

Figure 1: Inhibition of transactivation activity of HNF-4α by wild-type and mutant SHP proteins. It has been shown previously that expression of wild-type SHP significantly decreases HNF-4α transactivation of the HNF-1α gene prompter in HepG2 cells, indicating that SHP is a negative regulator of HNF-4α (Nishigori et al., 2001, Lee et al., 2000). Transcriptional regulation of the novel six mutations of p.Arg38Cys, p.Arg45Pro, p.Arg54Cys, p.Val105Gly, p.Asp178Asn and p.Trp206X was examined by luciferase reporter assay (n=3 in each experiment). Functional properties of the other mutations identified have been examined previously (Nishigori et al., 2001, Echwald et al., 2004). The relative luciferase activity (firefly/Renilla) of each construct at 0 ng, 25 ng, and 125 ng of wild-type and mutant SHP proteins was measured in HepG2 cells. Percent activity in relation to basic HNF-4α activity is shown as mean ± SD. * $P<0.05$.

DISCUSSION

Mutations in the SHP gene have been shown to be associated with high birth weight and early-onset mild obesity in Japanese. Although the molecular mechanism by which these mutations increase body weight is unknown at present, one possibility is suggested by the fact that pancreatic β cells express SHP mRNAs at high levels. Since SHP inhibits HNF-4α (MODY1 protein) (Nishigori et al., 2001,Lee et al., 2000), functional defects of SHP might well increase the activity of HNF-4α and other downstream components of glycolytic signal transduction (Dukes et al., 1998), resulting in increased insulin secretory response to glucose (Wang et al., 2006). In addition, since insulin is a key hormone in fetal growth, high levels of fetal insulin may well be associated with high birth weight and postnatal obesity.

As adult-onset type 2 diabetes is a polygenic disorder requiring interaction of multiple genetic and environmental factors, and Japanese patients exhibit a lesser insulin secretory capacity due to pancreatic β–cell

dysfunction (Kosaka et al., 1977, Kosaka and Akanuma, 1980, Yoshinaga and Kosaka, 1999), the increased insulin secretory demand associated with *SHP* mutations might increase susceptibility to type 2 diabetes in this population. Since other nuclear receptors that interact with SHP in peripheral tissues (Seol et al., 1996, Masuda et al., 1997, Seol et al., 1998, Johansson et al., 1999) may be involved in the pathogenesis of insulin resistance due to obesity or fatty liver, the secondary demand for compensatory insulin secretion might also promote the development of overt diabetes.

FXR-null mice, which show reduced levels of SHP, exhibit elevated plasma cholesterol and triglyceride levels and excessive accumulation of fat in the liver (Ma et al., 2006). Fatty liver also was observed in some early-onset obesity patients with *SHP* mutations (Nishigori et al., 2001). In addition, increased insulin secretion derived from *SHP* mutations accelerates fat accumulation in the liver. Accordingly, we examined 93 NASH patients, and found one mutation. However, none of the mutations associated with reduced activity was found in the NASH group, suggesting that the effect of SHP on the accumulation of fat in the liver may be of little clinical importance.

While we cannot link the etiology of NASH to mutation of *SHP*, the finding that *SHP* mutations increase morbidity risk for type 2 diabetes due to mild obesity in later life in Japanese suggests a genetic link between obesity and type 2 diabetes in human. To clarify the complex relationship of type 2 diabetes with SHP deficiency, further genetic analysis and characterization of the diabetogenic factors involved is required.

According to previous epidemiological studies, low birth weight and fetal thinness are associated with insulin resistance syndrome and were therefore thought to be related to later risk of type 2 diabetes (Hales et al., 1991, Eriksson et al., 2003). In contrast, we demonstrate here an increased morbidity risk of type 2 diabetes due to *SHP* mutations associated with high birth weight and mild obesity in Japanese children. Further analysis of the functional properties of mutant SHP proteins in energy expenditure should provide new insight into the relationship between birth weight and the development of type 2 diabetes.

ACKNOWLEDGMENTS

We thank Tsuchida H., Yokoyama K., Uda I., and Ibe Y. for technical assistance.

REFERENCES

Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. 1999. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol 94: 2467-2474

Dukes ID, Sreenan S, Roe MW, Levisetti M, Zhou YP, Ostrega D, Bell GI, Pontoglio M, Yaniv M, Philipson L, Polonsky KS. 1998. Defective pancreatic b-cell glycolytic signaling in hepatocyte nuclear factor-1α-deficient mice. J Biol Chem 273: 24457-24464

Echwald SM, Andersen KL, Sorensen TIA, Larsen LH, Andersen T, Tonooka N, Tomura H, Takeda J, Pedersen O. 2004. Mutation analysis of NR0B2 among 1545 Danish men identifies a novel c. 278 G>A (p. G93D) variant with reduced functional activity. Hum Mutat 24: 381-387

Eriksson JG, Forsen T, Tuomilehto J, Osmond C, Barker DJP. 2003. Early adiposity rebound in childhood and risk of type 2 diabetes in adult life. Diabetologia 46: 190-194

Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C, Winter PD. 1991. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 303: 1019-1022

Hung CC, Farooqi IS, Ong K, Luan J, Keogh JM, Pembrey M, Teo GSH, Dunger D, Wareham NJ, O'Rahilly S. 2003. The contribution of variants in the small heterodimer partner gene to birthweight, adiposity and insulin levels: Mutational analysis and association studies in multiple populations. Diabetes 52:1288-1291

Japanese Society for the Study of Obesity. 2000. Committee Report. J Jpn Soc Study Obes 6: 18-28

Johansson L, Thomsen JS, Damdimopoulos AE, Spyrou G, Gustafsson J-A, Treuter E. 1999. The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERα and ERβ. J Biol Chem 274: 345-353

Kosaka K, Hagura R, Kuzuya T. 1977. Insulin responses in equivocal and definite diabetes, with special reference to subjects who had mild glucose intolerance but later developed definite diabetes. Diabetes 26: 944-952

Kosaka K, Akanuma Y. 1980. Heterogeneity of plasma IRI responses in patients with IGT. Diabetologia 18: 347-348

Lee Y-K, Dell H, Dowhan DH, Hadzopoulou-Cladaras M, Moore DD. 2000. The orphan nuclear receptor SHP inhibits hepatocyte nuclear factor 4 and retinoid X receptor transactivation: Two mechanisms for repression. Mol Cell Biol 20: 187-195

Ma K, Saha PK, Chan L, Moore DD. 2006. Farnesoid X receptor is essential for normal glucose homeostasis. J Clin Invest 116: 1102-1109

Masuda N, Yasuno H, Tamura T, Hashiguchi N, Furusawa T, Tsukamoto T, Sadano H, Osumi T. 1997. An orphan nuclear receptor lacking a zinc-finger DNA-binding domain: interaction with several nuclear receptors. Biochim Biophys Acta 1350: 27-32

Mitchell SM, Weedon MN, Owen KR, Shields B, Wilkins-Wall B, Walker M, McCarthy MI, Frayling TM, Hattersley AT. 2003. Genetic variation in the small heterodimer partner gene and young-onset type 2 diabetes, obesity, and birth weight in U.K. subjects. Diabetes 52: 1276-1279

Nishigori H, Tomura H, Tonooka N, Kanamori M, Yamada S, Sho K, Inoue I, Kikuchi N, Onigata K, Kojima I, Kohama T, Yamagata K, Yang Q, Matsuzawa Y, Miki T, Seino S, Kim M-Y, Choi H-S, Lee Y-K, Moore DD, Takeda J. 2001. Mutations in the small heterodimer partner gene are associated with mild obesity in Japanese subjects. Proc Natl Acad Sci USA 98: 575-580

Ono M, Saibara T. 2006. Clinical features of nonalcoholic steatohepatitis in Japan: evidence from the literature. J Gastroenterol 41: 725-732

Polonsky KS. 2000. Dynamics of insulin secretion in obesity and diabetes. Int J Obes Relat Metab Disord (Suppl.) 2: S29-31

Reaven GM. 1988. Role of insulin resistance in human disease. Diabetes 37: 1595-1607

Seol W, Choi H-S, Moore DD. 1996. An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. Science 272: 1336-1339

Seol W, Hanstein B, Brown M, Moore DD. 1998. Inhibition of Estrogen receptor action by the orphan receptor SHP (short heterodimer partner). Mol Endo 12: 1551-1557

Wang L, Huang J, Saha P, Kulkarni RN, Hu M, Kim YD, Park KG, Chan L, Rajan A, Lee I, Moore DD. 2006. Orphan receptor SHP is an important mediator of glucose homeostasis. Mol Endocrinol 20: 2671-81

WHO (Western Pacific Region) and IASO (The International Association for the Study of Obesity) /IOTF (The International Obesity Task Force). 2000. The Asia-Pacific perspective: Redefining obesity and its treatment. Web site URL; http://www.diabetes.com.au/pdf/obesity_report.pdf

Yoshinaga H, Kosaka K .1999. Heterogeneous relationship of early insulin response and fasting insulin level with development of non-insulin-dependent diabetes mellitus in non-diabetic Japanese subjects with or without obesity. Diabetes Res Clin Pract 44: 129-136