

Nutrient control of phosphorylation and translocation of Foxo1 in C57BL/6 and *db/db* mice

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Abstract. The nutrient response mediated by feeding or fasting plays an important role in controlling gluconeogenic gene expression such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxylase (PEPCK). The FOXO family of forkhead transcription factor Foxo1 (mouse FOXO1) is a key regulator that stimulates the expression of gluconeogenic genes in the nucleus but is phosphorylated by Akt (also known as protein kinase B; PKB) and translocated to the cytoplasm in response to insulin. Although it has been widely accepted that the cellular signaling of insulin represses Foxo1 function through Akt-dependent phosphorylation, the molecular mechanism behind the modulation of Foxo1 function by nutrient responses, including feeding or fasting, remains unknown *in vivo*. We investigated the consequences of the nutritional changes in Akt-mediated Foxo1 phosphorylation and translocation in the liver using control C57BL/6 and diabetic *db/db* mice. We found that feeding promotes the phosphorylation and nuclear exclusion of Foxo1, whereas fasting counteracted them in C57BL/6 mice. Notably, *db/db* mice exhibited constitutive phosphorylation but dominant nuclear accumulation of Foxo1, even though CREB phosphorylation usually occurred in the fasted status. Furthermore, in contrast to C57BL/6 mice, the expression of G6Pase, PEPCK and PGC-1 α genes during feeding was not down-regulated in *db/db* mice. Thus, we suggest that the accurate regulation of Foxo1 via Akt-dependent phosphorylation is required for physiological adaptation to different nutritional statuses.

Introduction

Hepatic gluconeogenesis is strictly controlled by the activities of two rate-limiting enzymes in the liver, glucose-6-phosphatase

(G6Pase) and phosphoenolpyruvate carboxy-kinase (PEPCK). The genes encoding these proteins are regulated at the transcriptional level by key hormones, which are precisely secreted in response to various nutritional statuses mediated by fasting or feeding (1). In the fasted status, glucagon is secreted and robustly stimulates gluconeogenesis to maintain blood glucose levels within a basal range while, in the fed status, insulin markedly increases and powerfully suppresses gluconeogenesis to protect the body against hyperglycemia. On the other hand, aberrant hormonal responses to the nutritional statuses are implicated in the pathogenesis of type 2 diabetes, producing excessive hepatic glucose and resulting in fasting hyperglycemia and exaggerated postprandial hyperglycemia (2).

Recent studies have revealed that the forkhead transcription factor, Foxo1, is an important target for mediating the effects of insulin on the gene expression of G6Pase and PEPCK downstream from phosphatidylinositol 3-kinase and Akt (3-6). Accordingly, we demonstrated that Foxo1 regulates peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) promoter activity, which plays a major role in mediating hepatic gluconeogenesis in response to starvation (7). In response to insulin or several growth factors, Foxo1 is phosphorylated at Thr-24, Ser-253 and Ser-316 by Akt, leading to nuclear exclusion (8-10) and subsequent degradation through SCF^{Skp2}-mediated ubiquitination (11-14). In addition to phosphorylation, we and others have shown that reversible acetylation of Foxo1 mediated by CBP/p300 and Sir2/SIRT1 is involved in modulating its transactivation function (15-20). Furthermore, our previous report has found that acetylation of Foxo1 weakens its DNA binding and facilitates PKB-dependent phosphorylation at Ser-253 (21).

From a physiological point of view, a genetic analysis with gain- and loss-of-function alleles has identified Foxo1 as a key modulator of hepatic gluconeogenesis under the insulin-Akt signaling pathway (22). Supporting this finding, hepatic production of Foxo1- Δ 256, mutant of which interferes with Foxo1 function, rescues a diabetic phenotype in *db/db* mice by reducing G6Pase gene expression (23). Thus, it is well established that Foxo1 plays a critical role in mediating the effects of insulin on glucose metabolism (24), but how the nutrient responses due to fasting or refeeding control the Foxo1 function remains unsolved.

In this study, we sought to address two issues; the first is whether the endogenous Foxo1 protein is actually regulated by the nutrient response, the second is whether the nutritional

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regulation of Foxo1 is also observed in diabetic mice. To this end, we examined the phosphorylation levels and intracellular localization of Foxo1 during *ad libitum* feeding, fasting or refeeding in control C57BL/6 and diabetic *db/db* mice. We demonstrated that refeeding induced Akt-dependent phosphorylation and nuclear exclusion of Foxo1 in C57BL/6 mice. Conversely, constitutive phosphorylation but dominant nuclear accumulation of Foxo1 was observed throughout each nutritional status in *db/db* mice. Moreover, *db/db* mice did not exhibit the down-regulation of G6Pase, PEPCCK and PGC1 α gene expression, even in the fed status. These results support the notion that finely tuned phosphorylation of Foxo1 function under different nutrient signaling, at least in part, contributes to glucose homeostasis.

Materials and methods

Animals. C57BL/6J and *db/db* male mice at 8 weeks of age were purchased from CLEA, Inc. (Osaka, Japan) and adapted to the environment for 2-3 weeks before initiation of the study. Animals were fed standard rodent diet and water *ad libitum* in sterile cages (one mouse per cage) maintained under a light/dark cycle of 12 h (lights on at 7:00). In the feeding experiment, mice were sacrificed at the status of fed *ad libitum* (18:00), starved for 16 h (10:00), or starved for 16 h followed by refeeding with a high-sucrose/fat-free diet (a potent insulin-stimulating diet formula) for 4 h (14:00). Care of experimental animals was within institutional guideline approved by the Laboratory Animal Resource Center at the University of Tsukuba.

Determination of glucose and insulin levels. For measurements of blood glucose and serum insulin, samples were obtained from the tails of mice at the status of fed *ad libitum*, fasted or refed. Blood glucose was measured using a OneTouch Assist (LifeScan, Inc.). Serum insulin concentration was measured with the mouse insulin ELISA (Morinaga, Inc.).

Preparation of whole tissue extracts. Animals were sacrificed and the removed livers were quickly frozen in liquid nitrogen. One hundred milligrams of liver was homogenized using a polytron on ice in 2.5 ml of the buffer containing 50 mM HEPES-KOH (pH 7.9), 1% Nonidet P-40, 250 mM KCl, 5 mM EGTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF and protease inhibitors. Samples were rotated for 1 h at 4°C and centrifuged at 14000 rpm for 20 min at 4°C. The supernatants were stored at -80°C until analysis. Total protein concentrations of the extracts were measured using a protein assay kit (Bio-Rad, Inc.).

Fractionation of cytoplasmic and nuclear proteins. Cytoplasmic and nuclear protein extracts were fractionated using NE-PER extraction reagents (Pierce, Inc.). The mouse livers were cut into small pieces and then homogenized using tissue homogenizer for preparation of single cells. Thereafter, packed cell (40 mg) by centrifugation at 500 rpm for 3 min at 4°C was homogenized in 400 μ l of the ice-cold CER-I solution (Pierce, Inc.) supplied with protease inhibitors. Subsequent steps for fractionation were performed according to the manufacturer's instructions.

Antibodies and Western blotting. A rabbit polyclonal antibody specific for mouse Foxo1 was described previously (7). Antibodies for phospho-Foxo1 (Thr-24), phospho-Foxo1 (Ser-256), Akt, phospho-Akt (Ser-473), CREB and phospho-CREB (Ser-133) were purchased from Cell Signaling Technology (Beverly, MA).

Protein extracts were separated by 8% SDS-PAGE, followed by electrotransfer onto PVDF membrane and probed with indicated first antibodies. Chemiluminescent detection relied on horseradish peroxidase-conjugated secondary antibodies. The intensity of the protein bands was quantified by densitometry using Image Quant software (Molecular Dynamics, Inc.) and expressed as the mean \pm SE.

Immunohistochemistry. The livers were fixed in 4% paraformaldehyde overnight at 4°C and then embedded in paraffin. The paraffin sections (4 μ m) on slides were deparaffinized in xylene (3 min, three times), and rehydrated in a decreasing ethanol series diluted in distilled water (100, 100, 90, 80 and 70%, 3 min each). We used a microwave-based antigen retrieval method to enhance the reactivity of an antibody as described previously (25). Endogenous peroxidase activity was inactivated by 3% hydrogen peroxide in methanol for 15 min. The sections were blocked with 2.5% normal goat serum in PBS containing 0.25% Triton X-100 for 30 min. The anti-Foxo1 (1:100 dilution) antibody was incubated with the sections at room temperature for 1 h in a humidity chamber. Peroxidase-based detection was performed using Dako's Envision system, followed by color reaction using DAB substrate. Pictures were obtained using an optical microscope (Leica, Inc.).

Total RNA extraction and Northern blot analysis. The livers were homogenized using a polytron and total RNA was isolated using the Isogen RNA isolation reagent (Nippon Gene, Inc.). Fifteen micrograms of total RNA were denatured with glyoxal and DMSO, separated by electrophoresis and transferred to a nylon membrane (NEN Life Science Products, Inc.). The membranes were hybridized with ³²P-labeled probes under stringent conditions. Hybridization products were detected by an image analyzer (Typhoon 8600, Amersham Biosciences, Inc.) and standardized against human β -actin bands. A probe for Foxo1 was generated by PCR using primers as follows: forward, 5'-gaattcaattcgccacaatctgtccc-3'; and reverse, 5'-ttagcctgacacccagctgtgtg-3'. A probe for SREPB-1c was prepared by RT-PCR using first-strand cDNA from mouse epididymal fat total RNA as previously described (26). Probes for G6Pase, PEPCCK and β -actin were prepared as described previously (27). The cDNA probe of PGC-1 α was kindly donated by Y. Kamei (28). The intensity of the RNA bands was quantified by densitometry using Image Quant software (Molecular Dynamics, Inc.) and expressed as the mean \pm SE.

Results

Effects of nutritional status on Akt-mediated Foxo1 phosphorylation. We first identified the characteristics of C57BL/6 and *db/db* mice subjected to *ad libitum* feeding, overnight fasting or the following refeeding. As shown in Table I, fasting actually reduced the concentrations of blood glucose and serum



SPANDIDOS PUBLICATIONS Blood glucose and serum insulin levels of control C57BL/6 and diabetic *db/db* mice.

	C57BL/6			<i>db/db</i>		
	Fed <i>ad libitum</i>	Fasted	Refed	Fed <i>ad libitum</i>	Fasted	Refed
Blood glucose (mg/dl)	151±12	83±7 ^a	143±8	571±18 ^c	317±36 ^{b,c}	544±31 ^c
Serum insulin (ng/ml)	0.94±0.04	0.56±0.05 ^a	1.45±0.07	14.2±1.4 ^c	6.4±0.74 ^{b,c}	22.1±1.2 ^c

^aP<0.05, significant difference compared to mice fed *ad libitum* and refed. ^bP<0.01, significant difference compared to mice fed *ad libitum* and refed. ^cP<0.001, significant difference compared to C57BL/6 mice in each nutritional status. Data represent the mean ± SE (n=4).

insulin, whereas refeeding increased them in both C57BL/6 and *db/db* mice. The blood glucose and serum insulin levels of *db/db* mice were significantly elevated compared with control C57BL/6 mice throughout the different nutritional statuses, indicating that *db/db* mice exhibit severe insulin resistance and hyperglycemia.

To verify whether endogenous Foxo1 is regulated by nutritional statuses *in vivo*, we examined the phosphorylation levels of Foxo1 at Thr-24 and Ser-253 residues, both of which are phosphorylated by Akt via the insulin-signaling pathway. We prepared liver extracts from C57BL/6 and *db/db* mice under conditions of *ad libitum* feeding, fasting or refeeding and analyzed the expression and phosphorylation levels of Foxo1 and Akt by Western blotting. Compared to *ad libitum*

feeding, overnight fasting attenuated the phosphorylation levels of Foxo1 at both Thr-24 and Ser-253, whereas refeeding restored these levels in C57BL/6 mice [Fig. 1A (lanes 1-3) and B]. It should be noted that the alteration of nutritional statuses showed no substantial effect on the amount of hepatic Foxo1 protein (Fig. 1A, lanes 1-3). To further assess whether the insulin-Akt-signaling pathway is also involved in the nutrient response, we examined the phosphorylation levels of Akt at Ser-473 under fasting or refeeding conditions in the liver. Consistent with the result of Foxo1, the phosphorylation level of Akt was attenuated during fasting, suggesting that the phosphorylation of Akt and Foxo1 is controlled under the different nutritional statuses, probably through the insulin-signaling pathway, in C57BL/6 mice.

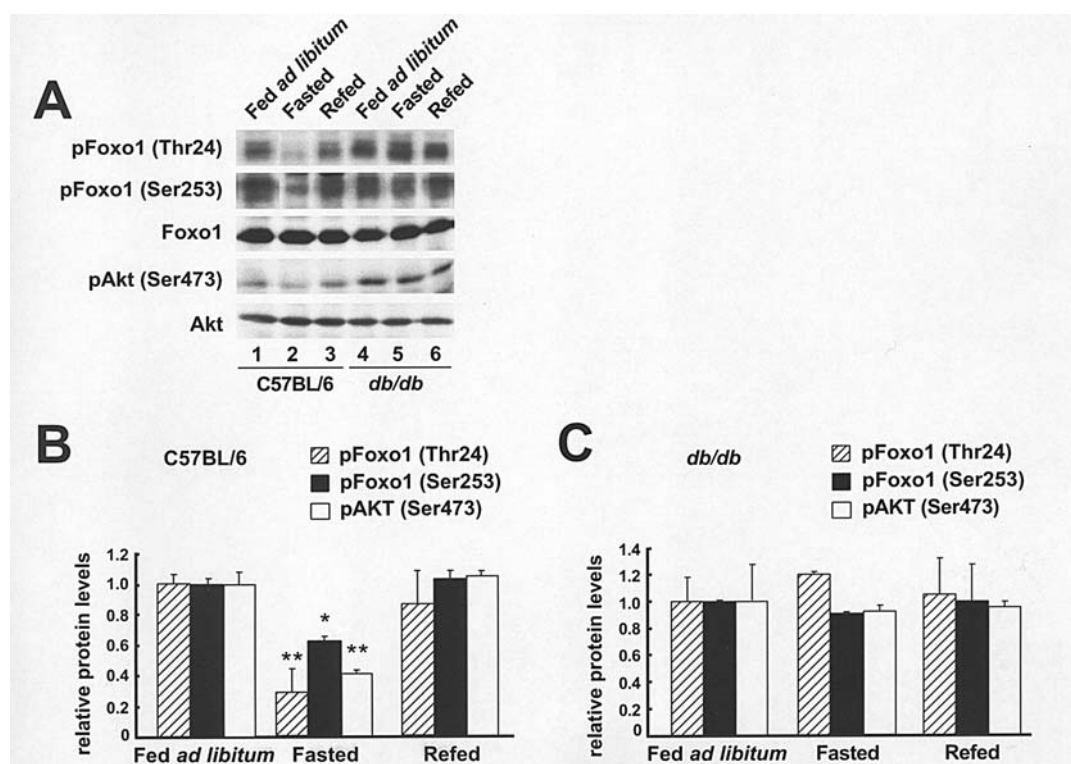


Figure 1. Effect of nutritional status on Akt-mediated phosphorylation of Foxo1 in C57BL/6 and *db/db* mice. (A) Liver extracts (50 µg) from C57BL/6 and *db/db* mice fed *ad libitum*, fasted or refed were immunoblotted by specific antibodies as indicated. A typical autoradiogram, representative of three independent experiments with similar results, is shown. (B and C) Quantification (relative value of fed *ad libitum* control) of phosphorylated Foxo1 (Thr-24, striped bar; Ser-253, black bar) and phosphorylated Akt (Ser-473, white bar) normalized to results from amount of Foxo1 and Akt in the indicated nutritional conditions of C57BL/6 (B) or *db/db* (C) mice. Each bar represents the mean ± S.E (n=3); *p<0.05, **p<0.01, compared to mice fed *ad libitum*.

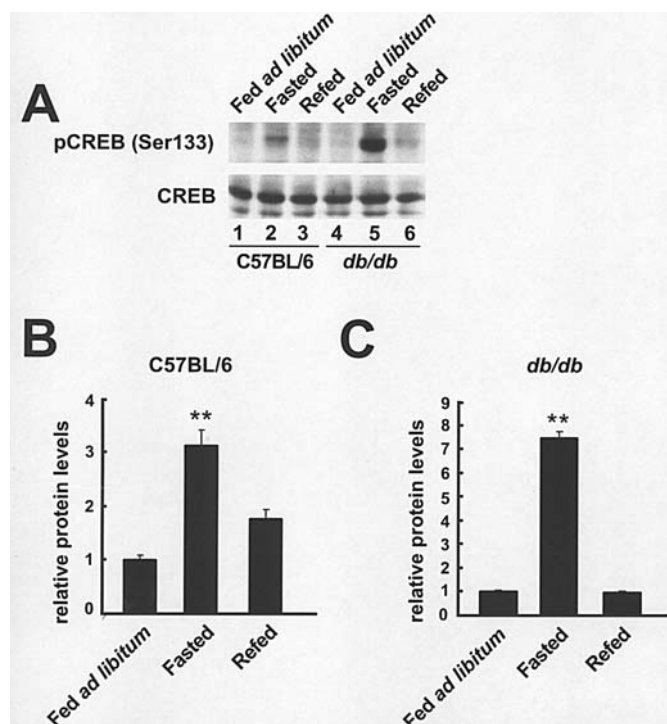


Figure 2. Effect of nutritional status on CREB phosphorylation in C57BL/6 and *db/db* mice. (A) Liver extracts (50 μ g) from C57BL/6 and *db/db* mice fed *ad libitum*, fasted or refed were immunoblotted using specific antibodies for CREB and phospho-CREB (Ser-133). A typical autoradiogram, representative of three independent experiments with similar results, is shown. (B and C) Quantification (relative value of fed *ad libitum* control) of phosphorylated CREB normalized to the amount of CREB in the indicated nutritional conditions of C57BL/6 (B) or *db/db* (C) mice. Each bar represents the mean \pm SE (n=3); **p<0.01, compared to mice fed *ad libitum*.

Next, we employed *db/db* mice as a model of type 2 diabetes and investigated whether Foxo1 phosphorylation cascades could be normally transmitted by nutrient responses. Surprisingly, in contrast to the control C56BL/6 mice [Fig. 1A (lanes 4-6) and B], there were no nutritional changes in the phosphorylation levels of Foxo1 and Akt in *db/db* mice (Fig. 1A, lanes 4-6). Moreover, highly phosphorylated Foxo1 and Akt were sustained in *db/db* mice in all nutrient statuses compared with C57BL/6 mice (Fig. 1A). These findings suggest that *db/db* mice are deficient in the nutrient-induced phosphorylation of both Foxo1 and Akt in the liver.

Effects of nutritional status on CREB phosphorylation.

During a period of prolonged fasting, glucagon stimulates protein kinase A (PKA), which in turn phosphorylates the cyclic AMP (cAMP) response element binding (CREB) protein at Ser-133, thereby promoting gluconeogenesis through the induction of the nuclear receptor coactivator, PGC-1 α (29). To test whether the glucagon-signaling pathway responds to fasting, we monitored the phosphorylation levels of hepatic CREB protein in C57BL/6 and *db/db* mice. As expected, fasting increased CREB phosphorylation in C57BL/6 mice, whereas refeeding decreased it [Fig. 2A (lanes 1-3) and B]. In contrast to the constitutive phosphorylation of Foxo1 and Akt throughout each nutritional status [Fig. 1A (lanes 4-6) and C], *db/db* mice showed a marked increase in the level of CREB phosphorylation by approximately 7-fold in the fasted

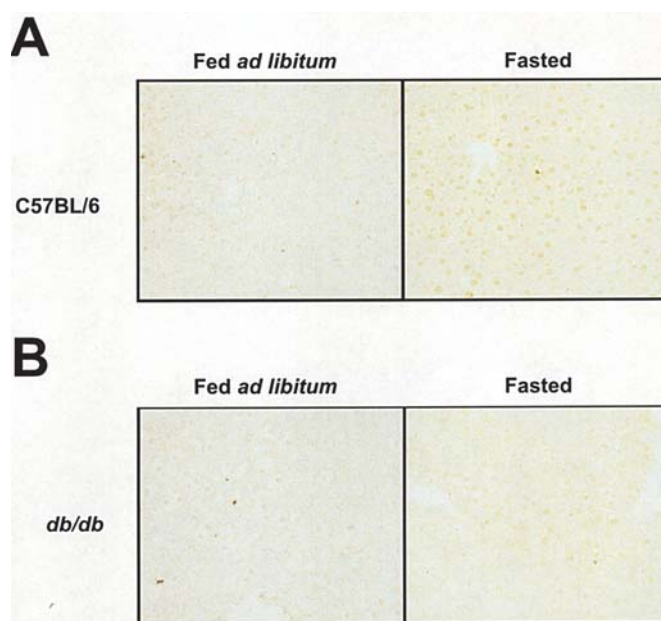


Figure 3. Hepatic immunohistochemistry of Foxo1 in C57BL/6 and *db/db* mice. Paraffin sections were prepared for immunohistochemical staining with anti-Foxo1 antibody. Positive staining for Foxo1 is brown. Original magnification is x400. Liver sections from C57BL/6 (A) and *db/db* (B) mice fed *ad libitum* (left panels) and fasted (right panels).

status [Fig. 2A (lanes 4-6) and C]. Moreover, a drastic effect of fasting on CREB phosphorylation was also observed in *db/db* mice compared with C57BL/6 mice (Fig. 2A). These data indicate that the nutrient response is usually transmitted to CREB, probably via the glucagon-PKA signaling pathway, even in *db/db* mice.

Effects of nutritional status on Foxo1 localization. Since the insulin/Akt-mediated phosphorylation of Foxo1 has been shown to correlate with its intracellular localization in mammalian cultured cells (8-10), we investigated whether Foxo1 could be also regulated by nutritional statuses in the mouse liver using immunohistochemical assay. As shown in Fig. 3A, fasting caused an extensive nuclear accumulation of Foxo1 in C57BL/6 mice. In contrast, Foxo1 predominantly localized in the nucleus in both fed *ad libitum* and fasted *db/db* mice (Fig. 3B).

To further support the above data, we performed Western blot analysis using cytoplasmic and nuclear fractionations of the liver extracts. In C57BL/6 mice, the amount of cytoplasmic Foxo1 protein was decreased by fasting compared to the fed *ad libitum* and refed statuses, while the nuclear Foxo1 was increased by fasting compared with the feeding statuses (Fig. 4A, lanes 1-3). Furthermore, the Foxo1 level in the ratio of nucleus to cytoplasm significantly increased by fasting (Fig. 4B). These data suggested that the intracellular localization of Foxo1 is tightly controlled in response to nutrient responses. In agreement with the results from immunohistochemistry, there were no nutritional changes in the amounts of nuclear and cytoplasmic Foxo1 proteins or in their ratio of nucleus to cytoplasm in *db/db* mice [Fig. 4A, (lanes 4-6) and C]. Collectively, these findings indicate that feeding stimulates the nuclear exit of Foxo1 in the liver, but this nutrient response to Foxo1 translocation is impaired in *db/db* mice.

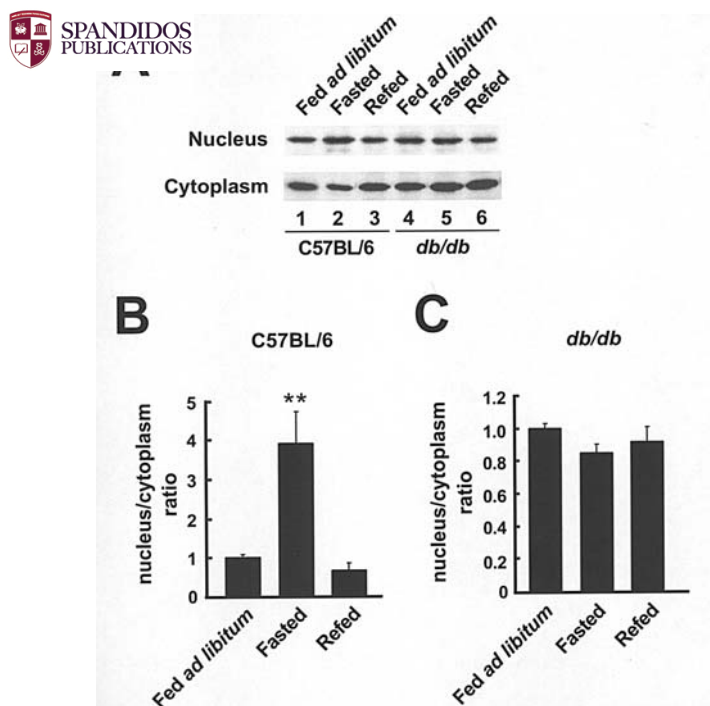


Figure 4. Intracellular distribution of Foxo1 in response to nutritional status. (A) Cytoplasmic and nuclear protein extracts (100 μ g) from C57BL/6 and *db/db* mice fed *ad libitum*, fasted or refed were immunoblotted by anti-Foxo1 antibody. A typical autoradiogram, representative of three independent experiments with similar results is shown. (B and C) Quantification (relative value of fed *ad libitum* control) of the Foxo1 level in the ratio of nucleus to cytoplasm in the indicated nutritional conditions of C57BL/6 (B) or *db/db* (C) mice. Each bar represents the mean \pm SE (n=3); **p<0.01, compared to mice fed *ad libitum*.

Effects of nutritional status on Foxo1-mediated transcription.

To examine the effects of nutrient response on the transactivation function of Foxo1, we evaluated the expression levels of the Foxo1-mediated genes, G6Pase, PEPCK and PGC-1 α , under the different nutritional statuses. Consistent with the result from the nucleus/cytoplasm ratio of Foxo1 (Fig. 4B), the mRNA levels of these gluconeogenic genes were substantially induced in the fasted status and then entirely diminished by refeeding in C57BL/6 mice [Fig. 5A (lanes 1-3) and B]. However, the mRNA level of sterol regulatory element binding protein-1c (SREBP-1c), which is a transcriptional regulator for lipid synthesis and is transactivated during the fed status (30,31), showed the opposite response to nutritional status. Notably, fasting-dependent repression of SREBP-1c mRNA was observed in both C57BL/6 and *db/db* mice (Fig. 5). In contrast, neither fasting nor refeeding changed the expression levels of G6Pase, PEPCK and PGC-1 α in *db/db* mice [Fig. 5A (lanes 4-6) and C], suggesting that *db/db* mice are also deficient in the nutrient responses to gluconeogenic gene expression as well as the deregulation of Akt-mediated Foxo1 phosphorylation and translocation.

Discussion

The present study demonstrates a direct correlation between nutritional status and the transactivation function of Foxo1 in mouse liver. We have shown that fasting drastically decreases the phosphorylation level of Foxo1, whereas refeeding markedly increases it, thereby promoting the nuclear exclusion of Foxo1 (Figs. 1, 3 and 4). Accompanied by the phosphoryl-

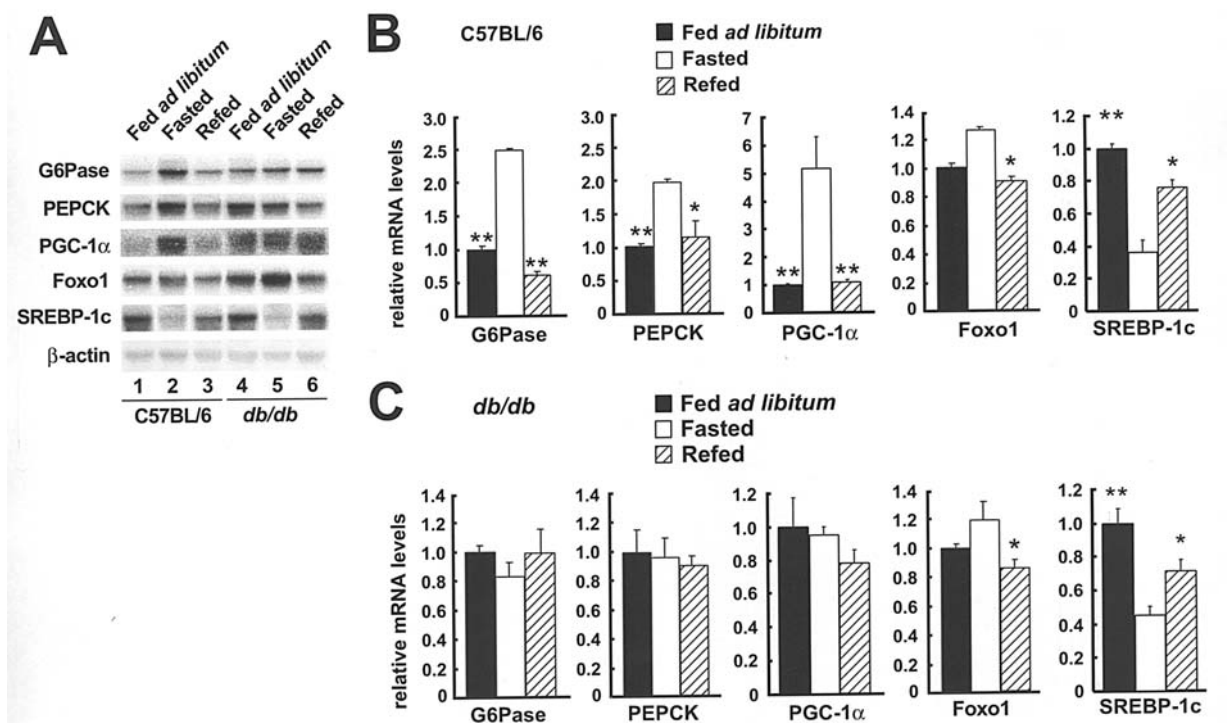


Figure 5. Nutritional regulation of the expression of the G6Pase, PEPCK, PGC-1 α , Foxo1 and SREBP-1c genes in C57BL/6 and *db/db* mice. (A) Northern blot analysis of G6Pase, PEPCK, PGC-1 α , Foxo1, SREBP-1c and β -actin mRNA in the livers of C57BL/6 and *db/db* mice fed *ad libitum*, fasted or refed. (B and C) Quantification (relative value of fed *ad libitum* control) of Foxo1, G6Pase, PEPCK PGC-1 α and SREBP-1c gene expression normalized to β -actin in the indicated nutritional conditions of C57BL/6 (B) or *db/db* (C) mice. Fed *ad libitum*, black bar; fasted, white bar; refed, striped bar. Each bar represents the mean \pm SE (n=3); *p<0.05, **p<0.01, compared to fasted mice.

ation and intracellular translocation of Foxo1, refeeding reduces the expression levels of G6Pase, PEPCK and PGC-1 α (Fig. 5). These results lead us to conclude that the metabolic information on physiological changes due to feeding or fasting is converted into the phosphorylation of Foxo1 via the Akt-dependent pathway, and the extent of phosphorylation represents the expression levels of gluconeogenic genes in the liver.

An alternative finding of this work is that diabetic *db/db* mice showed aberrant nutrient control of Foxo1, namely its constitutive phosphorylation and nuclear accumulation throughout each nutritional status (Figs. 1, 3 and 4). Supporting these results from *db/db* mice, we also found impaired nutrient control of Akt and Foxo1 in another diabetic model, New Zealand Obese mice (data not shown). Altomonte *et al* (23) have reported that *db/db* mice exhibit a significant induction of hepatic Foxo1 protein as well as its increased nuclear localization, compared with their heterozygous littermates (*db/+*). Considering this finding along with our present results, a plausible explanation for excessive hepatic gluconeogenesis in *db/db* mice may be an abnormal regulation of Foxo1 in both transcriptional and post-translational levels.

Recent studies suggested that Akt activity was decreased in adipose tissue and skeletal muscle in *db/db* mice (32,33). In contrast, we found that continuous Ser473-phosphorylated Akt, which equates to greater insulin signaling, was even maintained in fasting conditions in *db/db* mice. This result does not correspond with an explanation for insulin resistance. Although we could not clarify the mechanism whereby phosphorylated Akt was retained in the *db/db* mice, Clodfelder-Miller *et al* (34) have shown that the phosphorylation level of brain Akt in *db/db* mice was higher than in control C57BL/6 mice, suggesting that a glucose-sensing mechanism regulates brain Akt activity. Thus, hyperglycemia in *db/db* mice may cause large increases in the phosphorylation levels of hepatic Akt and its substrate, Foxo1.

Despite the aberrant nutrient control of Akt and Foxo1 in *db/db* mice, we found that the nutrient response by feeding robustly correlates with the phosphorylation levels of CREB (Fig. 2). This finding strongly suggests that *db/db* mice possess an adequate response to glucagon, which in turn phosphorylates hepatic CREB protein through the PKA-signaling pathway. Given the constitutive hyper-phosphorylation and abnormal nuclear accumulation of Foxo1 in the liver of *db/db* mice, a contributing factor to hyperglycemia in diabetes appears to be an impaired suppression of Foxo1 transactivation function in response to feeding through the insulin-signaling pathway.

One significant question of our results is the mechanism whereby the nutritional conditions do not alter the intracellular localization of Foxo1 despite its hyper-phosphorylation status in *db/db* mice. Notably, a recent study has revealed that c-Jun N-terminal kinase (JNK) directly phosphorylates FOXO4, which leads to nuclear translocation and transcriptional activation (35). JNK has been shown as a mediator of oxidative stress induced by tumor necrosis factor α and hyperglycemia (36) and was revealed to be abnormally elevated in obesity and insulin resistance (37). In view of our present results, it is possible that immoderate JNK activity might override the Akt-mediated nuclear exclusion of Foxo1 and sustain its nuclear accumulation, resulting in the constitutive expression of gluconeogenic genes in *db/db* mice.

In conclusion, this study presents significant information on a critical role for Foxo1 in response to nutrient signaling. Impaired nutritional regulation of Akt-mediated Foxo1 phosphorylation and translocation could, at least in part, account for an uncontrolled expression of gluconeogenic genes. In addition to phosphorylation, we previously demonstrated that Foxo1 is acetylated by CBP, the modification of which is reversed by the NAD⁺-dependent histone deacetylase, Sir2 (20). It has recently been reported that SIRT1/Sir2 controls the hepatic gluconeogenic/glycolytic pathways in response to fasting through the transcriptional coactivator, PGC-1 α (38). Given that SIRT1 protein levels and NAD⁺ concentration increase in the fasted status (38), it is conceivable that the nutrient signaling including fasting or refeeding alters the levels of Foxo1 acetylation via Sir2/SIRT1-mediated deacetylation in the liver. Further studies are needed to clarify an alteration of the levels of acetylation as well as phosphorylation of Foxo1 in response to nutritional status.

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References

1. Nardlie RC, Foster JD and Lange AJ: Regulation of glucose production by the liver. *Annu Rev Nutr* 19: 379-406, 1999.
2. Staehr P, Hother-Nielsen O and Beck-Nielsen H: Hepatic glucose production: therapeutic target in type 2 diabetes? *Diabetes Obes Metab* 4: 215-223, 2002.
3. Schmoll D, Walker KS, Alessi DR, Grempler R, Burchell A, Guo S, Walther R and Unterman TG: Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity. *J Biol Chem* 275: 36324-36333, 2000.
4. Nakae J, Kitamura T, Silver DL and Accili D: The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* 108: 1359-1367, 2001.
5. Hall RK, Yamasaki T, Kucera T, Waltner-Law M, O'Brien R and Granner DK: Regulation of phosphoenolpyruvate carboxy-kinase and insulin-like growth factor-binding protein-1 gene expression by insulin. The role of winged helix/forkhead proteins. *J Biol Chem* 275: 30169-30175, 2000.
6. Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D and Spiegelman BM: Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1 alpha interaction. *Nature* 423: 550-555, 2003.
7. Daitoku H, Yamagata K, Matsuzaki H, Hatta M and Fukamizu A: Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* 52: 642-649, 2003.
8. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J and Greenberg ME: Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96: 857-868, 1999.
9. Nakae J, Barr V and Accili D: Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR. *EMBO J* 19: 989-996, 2000.



SPANDIDOS: Heide LP, Hoekman MF and Smidt MP: The ins and outs of transcriptional regulation. *Biochem J* 380: 297-309, 2004.

11. Huang H, Regan KM, Wang F, Wang D, Smith DI, van Deursen JM and Tindall DJ: Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci USA* 102: 1649-1654, 2005.
12. Matsuzaki H, Daitoku H, Hatta M, Tanaka K and Fukamizu A: Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci USA* 100: 11285-11290, 2003.
13. Plas DR and Thompson CB: Akt activation promotes degradation of tuberlin and FOXO3a via the proteasome. *J Biol Chem* 278: 12361-12366, 2003.
14. Aoki M, Jiang H and Vogt PK: Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci USA* 101: 13613-13617, 2004.
15. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW and Greenberg ME: Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 303: 2011-2015, 2004.
16. Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, McBurney M and Guarente L: Mammalian SIRT1 represses forkhead transcription factors. *Cell* 116: 551-563, 2004.
17. Accili D and Arden KC: FoxOs at the crossroads of cellular metabolism, differentiation and transformation. *Cell* 117: 421-426, 2004.
18. Frescas D, Valenti L and Accili D: Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenic genes. *J Biol Chem* 280: 20589-20595, 2005.
19. Giannakou ME and Partridge L: The interaction between FOXO and SIRT1: tipping the balance towards survival. *Trends Cell Biol* 14: 408-412, 2004.
20. Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M, Nakajima T and Fukamizu A: Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci USA* 101: 10042-10047, 2004.
21. Matsuzaki H, Daitoku H, Hatta M, Aoyama H, Yoshimochi K and Fukamizu A: Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *Proc Natl Acad Sci USA* 102: 11278-11283, 2005.
22. Nakae J, Biggs WH III, Kitamura T, Caveness WK, Wright CV, Arden KC and Accili D: Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* 32: 245-253, 2002.
23. Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, Meseck M, Accili D and Dong H: Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. *Am J Physiol Endocrinol Metab* 285: E718-E728, 2003.
24. Barthel A, Schmolli D and Unterman TG: FoxO proteins in insulin action and metabolism. *Trends Endocrinol Metab* 16: 183-189, 2005.
25. Ye H, Holterman AX, Yoo KW, Franks RR and Costa RH: Premature expression of the winged helix transcription factor HFH-11B in regenerating mouse liver accelerates hepatocyte entry into S phase. *Mol Cell Biol* 19: 8570-8580, 1999.
26. Cederberg A, Gronning LM, Ahren B, Tasken K, Carlsson P and Enerback S: FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. *Cell* 106: 563-573, 2001.
27. Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J and Fukamizu A: Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *J Biol Chem* 279: 23158-23165, 2004.
28. Kamei Y, Miura S, Suzuki M, Kai Y, Mizukami J, Taniguchi T, Mochida K, Hata T, Matsuda J, Aburatani H, Nishino I and Ezaki O: Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes and impaired glycemic control. *J Biol Chem* 279: 41114-41123, 2004.
29. Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B and Montminy M: CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 413: 179-183, 2001.
30. Shimano H: Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog Lipid Res* 40: 439-452, 2001.
31. Ide T, Shimano H, Yahagi N, Matsuzaka T, Nakakuki M, Yamamoto T, Nakagawa Y, Takahashi A, Suzuki H, Sone H, Toyoshima H, Fukamizu A and Yamada N: SREBPs suppress IRS-2-mediated insulin signalling in the liver. *Nat Cell Biol* 6: 351-357, 2004.
32. Shao J, Yamashita H, Qiao L and Friedman JE: Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Leprdb/db mice. *J Endocrinol* 167: 107-115, 2000.
33. Yasukawa T, Tokunaga E, Ota H, Sugita H, Martyn JA and Kaneki M: S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance. *J Biol Chem* 280: 7511-7518, 2005.
34. Clodfelder-Miller B, De Sarno P, Zmijewska AA, Song L and Jope RS: Physiological and pathological changes in glucose regulate brain Akt and glycogen synthase kinase-3. *J Biol Chem* 280: 39723-39731, 2005.
35. Essers MA, Weijzen S, De Vries-Smits AM, Saarloos I, De Ruiter ND, Bos JL and Burgering BM: FOXO transcription factor activation by oxidative stress mediated by the small GTPase Rac and JNK. *EMBO J* 23: 4802-4812, 2004.
36. Bennett BL, Satoh Y and Lewis AJ: JNK: a new therapeutic target for diabetes. *Curr Opin Pharmacol* 3: 420-425, 2003.
37. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M and Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420: 333-336, 2002.
38. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM and Puigserver P: Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 434: 113-118, 2005.