Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation

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A forkhead-type transcription factor, DAF-16, is located in the most downstream part of the insulin signalling pathway via PI3K (phosphoinositide 3-kinase). It is essential for the extension of life-span and is also involved in dauer formation induced by food deprivation in *Caenorhabditis elegans*. In the present study, we addressed whether or not FOXO members AFX, FKHR (forkhead homologue in rhabdomyosarcoma) and FKHRL1 (FKHR-like protein 1), mammalian counterparts of DAF-16, are involved in starvation stress. We found a remarkable selective induction of FKHR and FKHRL1 transcripts in skeletal muscle of mice during starvation. The induction of FKHR gene expression was observed at 6 h after food deprivation, peaked at 12 h, and returned to the basal level by 24 h of refeeding. The induction was also found in skeletal muscle of mice with glucocorticoid treatment. Moreover, we found that the levels of PDK4 (pyruvate dehydrogenase kinase 4) gene expression were up-regulated through the direct binding of FKHR to the promoter region of the gene in C2C12 cells. These results suggest that FKHR has an important role in the regulation of energy metabolism, at least in part, through the up-regulation of PDK4 gene expression in skeletal muscle during starvation.

Key words: forkhead transcription factor, FOXO, gene regulation, pyruvate dehydrogenase kinase 4 (PDK4), skeletal muscle, starvation.

INTRODUCTION

Genes involved in the life-span extension of *Caenorhabditis elegans* have been clarified by mutant analysis, and the presence of a series of genes, including *daf-2, age-1* and *daf-16*, among them is intriguing because they are components of the insulin signalling pathway via PI3K (phosphoinositide 3-kinase), and they have counterparts in mammals [1]. In particular, a forkhead-type transcription factor DAF-16 is essential for life-span extension and acts in the most downstream part of the signalling pathway [1]. It is probable that the insulin or insulin-like hormone signalling pathway can have effects on the life-span through transcriptional regulation by DAF-16 counterparts, FOXO members [FKHR (forkhead homologue in rhabdomyosarcoma; FOXO1), FKHRL1 (FKHR-like protein 1; FOXO3) and AFX (FOXO4)] in mammals as well as in *C. elegans*. Moreover, the signalling pathway is also essential for dauer formation in *C. elegans*, which is a specific state induced by food deprivation that makes the nematodes highly resistant to stresses, such as starvation, UV and heat [2–4]. Therefore it is interesting to know whether or not FOXO members are involved in physiological responses to starvation stress in mammals. In addition, it is important to elucidate which kind of genes FOXO proteins control transcriptionally during starvation in order to understand the roles of FOXO genes.

There are several lines of evidence showing that FOXO members are involved in energy metabolism. For example, overexpressed FOXO proteins induce the gene expression of PEPCK (phosphoenolpyruvate carboxykinase) and G6P (glucose-6-phosphatase), which are key gluconeogenic and glycogenolytic enzymes, and their induction is inhibited by insulin in hepatocytes and kidney cell lines [5–9]. The stimulation by insulin leads to phosphorylation of FOXO protein by Akt activated through the insulin–PI3K pathway, and the protein is then translocated from the nucleus into the cytosol, resulting in reduced transcriptional activity [10,11]. It is reported that the levels of AFX and FKHR gene expression were higher in skeletal muscle and adipose tissues of mice respectively [12]. Moreover, it has recently been demonstrated that FKHR is involved in the differentiation of adipose tissues and obesity by a high-fat diet [13].

The decreased level of blood insulin during starvation can lead to the induction of PEPCK and G6P [14,15]. However, it is unclear whether the phosphorylation of FOXO proteins is actually the predominant mechanism for regulating their transcriptional activities during starvation. It is also possible that their activities are regulated by a change in the levels of FOXO proteins. Indeed, it has recently been reported that enhancement and inhibition of FKHR gene expression by oestrogen and gonadotropins occur in granulosa cells of developing follicles [16].

In the present study, we examined changes of the levels of FOXO genes expression in tissues involved in energy metabolism, i.e. liver, kidney, white adipose tissue and skeletal muscle, during starvation. We found that the expression of FKHR and FKHRL1 mRNA was remarkably increased in a selective manner in skeletal muscle by starvation and glucocorticoid treatment. Moreover, we identified PDK4 (pyruvate dehydrogenase kinase 4), an important enzyme regulating glucose consumption in skeletal muscle, liver and kidney, as a novel target gene of FKHR.

MATERIALS AND METHODS

Animals

C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). After 12-week-old male mice ($n = 8$) had been kept for 1 week

Abbreviations used: CR, caloric restriction; DBE, DAF-16 family binding element; DMEM, Dulbecco's modified Eagle's medium; FKHR, forkhead homologue in rhabdomyosarcoma; FKHRL1, FKHR-like protein 1; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; G6P, glucose-6-phosphatase; GMSA, gel mobility-shift assay; NEFA, non-esterified fatty acid; PDK4, pyruvate dehydrogenase kinase 4; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome-proliferator-activated receptor; Trx, thioredoxin; TBS, Tris-buffered saline.

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with *ad libitum* feeding, they were separated into two groups. One group was starved for 24 h, and the other group was fed *ad libitum*. They were then killed and the tissues (liver, kidney, the epididymal white adipose tissue and gastrocnemius muscle) were isolated for analysis of the expression of FOXO genes. To examine the time course of FOXO genes expression in gastrocnemius muscle, 12-week-old male C57BL/6 mice ($n = 15$) were separated into five groups. Groups ($n = 3$) were fed *ad libitum*, starved for 6, 12 or 24 h, or starved for 24 h followed by 24 h of refeeding. They were then killed and gastrocnemius muscle was isolated. The same experiment was repeated independently. For glucocorticoid treatment, 12-week-old male mice ($n = 8$) were kept for 1 week with intraperitoneal injection with 100 μl of sterile saline every day. One group was then injected with 100 μl of 10 mg/ml hydrocortisone succinate (Wako, Tokyo, Japan) suspended in saline, and another group was injected with saline alone as the control. After 6 h of treatment they were killed and their gastrocnemius muscles were taken for analysis. Animal care and all experiments were carried out according to the institutional guidelines of the National Institute for Longevity Sciences (NILS).

Cell culture

C2C12 cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 10 % foetal bovine serum (Sigma, St. Louis, MO, U.S.A.) in a 37 °C incubator with 5 % CO_2. For differentiation of the cells into myotubes, following confluence, the cells were transferred into a differentiation medium; i.e. DMEM with 2 % (v/v) horse serum.

Western blot analysis

Gastrocnemius muscles were homogenized in modified RIPA buffer [50 mM Tris/HCl, pH 7.4, containing 1 % (v/v) Nonidet P-40, 0.25 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml each of aprotinin, leupeptin and pepstatin, and 1 mM NaF) by using a Polytron probe (Tekmar, Cincinnati, OH, U.S.A.). Aliquots of protein (25 μg) were separated on 7.5 % (w/v) polyacrylamide reducing gels, at 20 mA for 90 min, and electroblotted on to Immobilon-P PVDF membranes (Millipore, Bedford, MA, U.S.A.). The blots were blocked with 5 % (w/v) BSA in TBS (Tris-buffered saline: 10 mM Tris/HCl, pH 7.4, and 150 mM NaCl) with 0.05 % (v/v) Tween 20 (TBS-T) for 60 min, and were then incubated with specific antibodies against FKHR (Upstate Biotechnology, Lake Placid, U.S.A.) and phospho-S256 FKHR (Cell Signaling Technology, Beverly, MA, U.S.A.) overnight at 4 °C. They were washed in TBS-T for 5 min and incubated with goat anti-sheep or goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody (diluted 1:2000) for 60 min at room temperature (20 °C). Then, the membranes were washed in TBS-T, and the bands were developed by ECL® (enhanced chemiluminescence) detection using a standard kit (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Northern blot analysis

Total RNAs were isolated from the tissues by using TRIzol® reagent (Gibco BRL). Northern blotting analysis was performed as previously described [12]. Blots were hybridized with specific probes labelled with [α-^{32}P]dCTP for the mRNAs of AFX, FKHR, FKHRL1, PDK4 and 18 S rRNA, and then exposed to films (Kodak, Rochester, NY, U.S.A.) or Bioimage analyser plates (Fuji Film, Tokyo, Japan). Hybridization signals were quantified by a Fuji Bioimage analyser and were normalized by 18 S rRNA or G3PDH (glyceraldehyde-3-phosphate dehydrogenase) by using a densitometric program, Image Gauge v. 3.11 (Fuji Film).

Adenovirus vector construction and infection

We isolated a fragment, FKHR-3A, which is a constitutively active mutant of FKHR, from pCDNA3.1-FKHR-3A as previously described [12]. We constructed AxCALNL-FKHR-3A by subcloning the fragment into pAxCALNLw Cre-loxP system-mediated expression cassette by using an Adenovirus Cre/loxP-Regulated Expression Vector Set (Takara, Otsu, Japan). AxCANCre was purchased from the RIKEN DNA bank (Tsukuba, Japan). C2C12 cells were infected using a standard technique. Briefly, C2C12 cells that have been in differentiation medium for 3 days were incubated in DMEM including the adenovirus for 1 h with seesawing for 30 s every 15 min. Then the medium was changed back to a differentiation medium.

Gel mobility-shift assay (GMSA)

Thioredoxin (Trx)-fused DNA-binding domain-deficient FKHR protein (ΔN-FKHR) was made from the vector pET32b-ΔN-FKHR by *Nco*I-mediated deletion from pET32b-FKHR-myc [12]. Bandshift analysis of the mouse PDK4 gene using Trx-FKHR-myc was performed as previously described [12]. The oligonucleotides used were mPDK4-DBEwt (5′-GGCTCTGG-AGTTTGTAAACAAGGACAAGTCTGGG-3′) and mPDK4-DBEmt (5′-GGCTCTGGAGTTTGTAGGGGAGGACAAGTC-TGGG-3′) with a mutation in DBE (DAF-16 family binding element), GTAAACAA [12]. They were labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Toyobo Biochemicals, Osaka, Japan). Competition experiments were performed with 1:100 ratios of labelled to non-labelled mPDK4-DBEwt or mPDK4-DBEmt oligonucleotides.

Luciferase assay

A 1 kb fragment of the promoter region of the mouse PDK4 gene was cloned from C57BL/6 tail genomic DNA by PCR using the forward primer 5′-CGCTAGCATGTTTCAACAGCA-GTCATTAGG-3′ and the reverse primer 5′-CGCTAGCATGTT-TCAACAGCAGTCATTAGG-3′. This fragment was subcloned into a pCRII vector, and a *Nhe*I/*Bam*HI fragment of the pCRII-mPDK4 promoter was ligated into *Nhe*I/*Bgl*II cut pGL3-basic vector (Promega, Madison, WI, U.S.A.) to create pGL3-mPDK4prom. The pGL3-mPDK4prom-DBEmt vector with a mutation in DBE was created by site-directed mutagenesis using the primer 5′-GGCTCTGGAGTTTGTAGGGGAGGACAAGT-CTGGG-3′. Underlined residues indicate the site of mutation. All constructs were verified by automated sequencing. Luciferase measurements were performed as previously described [12].

Statistical analysis

Data were analysed by one-way ANOVA. Fisher's PLSD test was used as a post-hoc test for multiple comparisons among samples in the time course of starvation and refeeding and in the reporter assay, and Student's t test was used for a comparison between control and test samples in glucocorticoid treatment using StatView 5.0 (SAS Institute, Cary, NC, U.S.A.). Data were presented as means ± S.E.M.

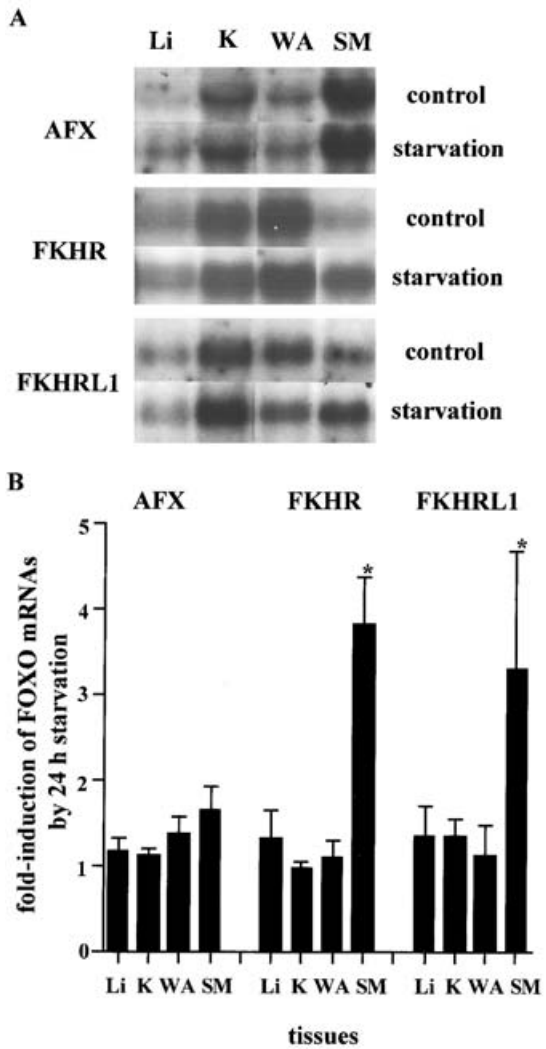

Figure 1 Changes in the levels of FOXO genes expression by 24 h of starvation

(**A**) Northern blot analysis for FOXO genes was performed by using total RNA from liver (Li), kidney (K), white adipose tissue (WA) and skeletal muscle (gastrocnemius) (SM) involved in energy metabolism of mice either starved for 24 h or fed *ad libitum* (control). Results are representative of four experiments, and the induction of FKHR and FKHRL1 genes in gastrocnemius muscle was remarkable. (**B**) Results in (**A**) were quantified and are means of fold-induction of each starvation group over the 0 h group ± S.E.M. ($n = 4$). *$P < 0.0001$. The 18 S rRNA was used as the intrinsic standard.

RESULTS

Changes in FOXO genes expression in mouse tissues caused by 24 h of starvation

Starvation for 24 h induced the expression of FKHR genes markedly and selectively in skeletal muscle among important tissues for energy metabolism, liver, kidney, white adipose tissues, and skeletal muscle (3.8-fold compared with the control in skeletal muscle, $P < 0.0001$) (Figure 1). The expression of AFX mRNA was high at the steady state in skeletal muscle and showed no remarkable change in skeletal muscle by starvation. The expression of FKHRL1 gene was also remarkably increased in skel–etal muscle (3.3-fold compared with the control, $P < 0.0001$). Thus we focused on the change in the expression of FOXO genes in skeletal muscle.

Changes in FOXO and PDK4 genes expression in skeletal muscle during starvation and refeeding

We examined the temporal changes of FOXO genes expression during starvation and after 24 h of refeeding (Figures 2A and 2B). The expression of AFX gene showed a small change during starvation and refeeding (1.5-fold at 24 h compared with 0 h, $P < 0.01$). The expression of the FKHR gene began to increase at 6 h (1.7-fold compared with 0 h, $P < 0.05$), peaked at 12 h (4.6-fold compared with 0 h, $P < 0.0001$) and remained at a significantly high level 24 h after food deprivation (3.8-fold compared with 0 h, $P < 0.0001$). The increased expression of the FKHR gene returned to the basal level by 24 h of refeeding. The induction of FKHRL1 gene expression by starvation was similar to that of FKHR, but was higher at 24 h than at 12 h (2.0-fold at 12 h, $P < 0.05$, and 3.3-fold at 24 h, $P < 0.0001$, compared with 0 h). In addition, we examined the change in PDK4 gene expression during starvation, because it is known that the expression of the gene is induced in skeletal muscle during starvation and diabetes [17,18]. An increase in the expression of PDK4 gene was detected 6 h after food deprivation (3.1-fold compared with 0 h, $P < 0.0001$), and was marked at 24 h (11.3-fold compared with 0 h, $P < 0.0001$). There was roughly a 12 h delay between the peak of induction of FKHR gene expression at 12 h and the marked induction of PDK4 gene at 24 h after food deprivation (Figures 2A and 2B). Hence we also examined temporal changes of the levels of FKHR and phospho-Ser-256 FKHR proteins (Figures 2C and 2D). The latter inversely correlates with transcriptional activities of FKHR [10,11]. The remarkable induction of FKHR protein was detected 24 h after food deprivation (2.5-fold compared with 0 h, $P < 0.0001$). On the other hand, the levels of phospho-Ser-256 FKHR also showed a slightly, but significant, change at 24 h after starvation (1.3-fold at 24 h compared with 0 h, $P < 0.05$).

Glucocorticoid induces FKHR gene expression in skeletal muscle

As the levels of blood glucocorticoid are increased during starvation and diabetes, we examined the possibility that glucocorticoid increases the expression of FOXO genes (Figure 3). Glucocorticoid treatment of mice for 6 h caused a remarkable increase in the expression of the FKHR gene (3.0-fold higher than control, $P < 0.05$). It also had a weaker effect on the levels of AFX and FKHRL1 gene expression (1.3- and 1.6-fold higher than control respectively, $P < 0.05$).

PDK4 is a target gene of FKHR

We examined whether or not FKHR induces the expression of the PDK4 gene, because a conserved consensus DBE is located at 362 and 359 nucleotides 5′ upstream of the transcriptional start site in the promoter region of mouse and human PDK4 genes respectively [19] (see Figure 5A). To verify that FKHR regulates the expression of the PDK4 gene, we examined the time course of PDK4 gene expression after infection with the adenovirus carrying FKHR-3A into C2C12 myotubes (Figure 4). We found that PDK4 gene expression was induced considerably at 12 h after infection when the exogenous FKHR protein began to be detected. The increase of PDK4 gene expression was dependent upon the expression level of FKHR-3A protein. However, the control adenovirus infection did not affect the expression of PDK4 gene. To assess the ability of FKHR to regulate the expression of PDK4 gene directly, we transfected C2C12 cells with an expression vector containing FKHR-3A together with a reporter vector, in which the PDK4 promoter drives the expression of a luciferase

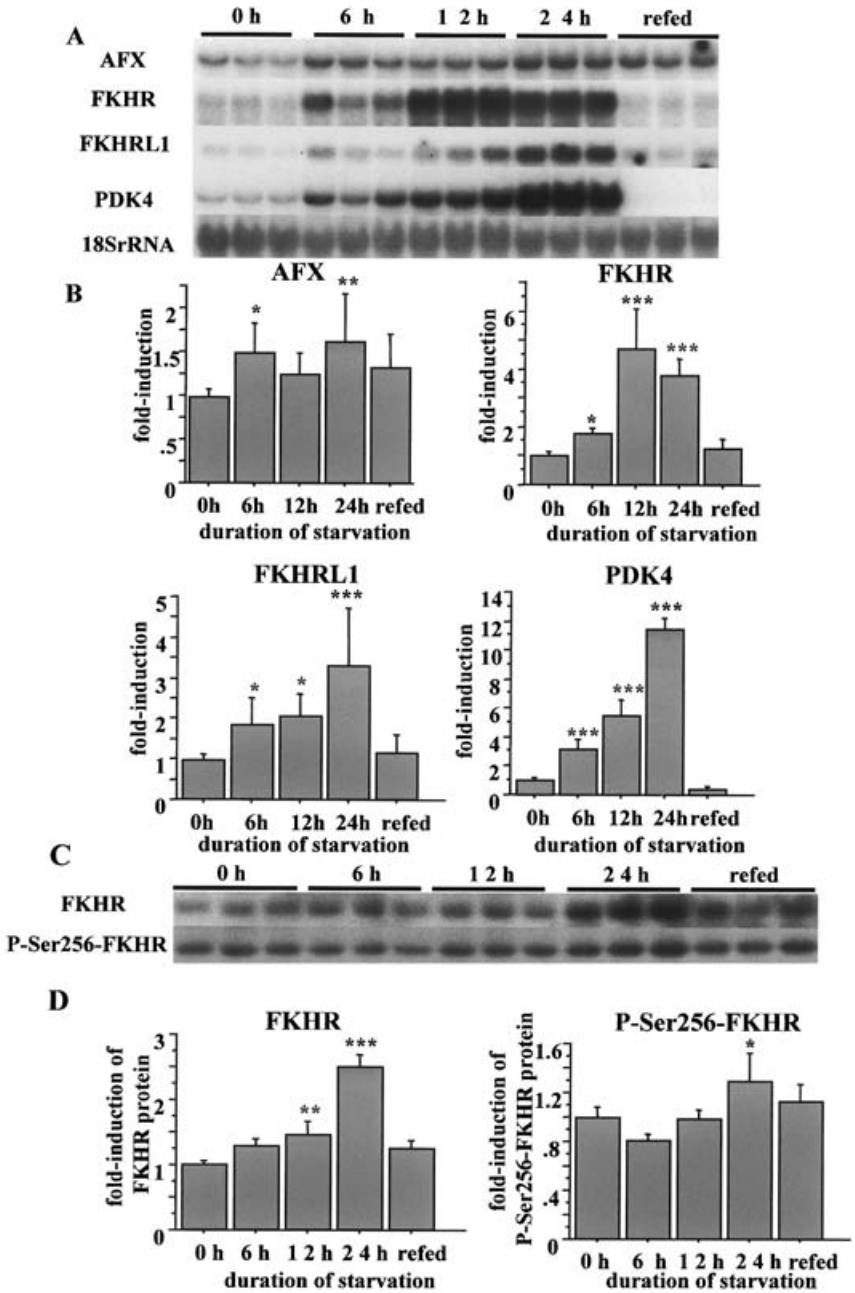

Figure 2 Temporal changes in the levels of FOXO genes expression in skeletal muscle during starvation and refeeding

(**A**) Northern blot analysis for FOXO and PDK4 genes was performed by using total RNAs from gastrocnemius muscle from mice fed *ad libitum*, starved for 6, 12 or 24 h, or refed for 24 h after 24 h of starvation. The intrinsic standard was 18 S rRNA. (**B**) Results in (**A**) were quantified and are means of fold-induction of each group over 0 h group ± S.E.M. ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.0001$ compared with the 0 h group. (**C**) Western blot analysis was performed by using proteins from a part of the gastrocnemius muscle from mice used in (**A**) and antibodies against FKHR and phospho-Ser-256-FKHR. (**D**) Results in (**C**) were quantified and are means of fold-induction of each group over the 0 h group ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.0001$ compared with the 0 h group.

gene (Figure 5B). We found that FKHR-3A effectively induced luciferase activity using the reporter vector with wild-type PDK4 promoter, but not with PDK4 promoter with mutation in the DBE. We then examined by GMSA whether or not the induction of the luciferase activity was derived from direct binding of FKHR protein to DBE in the promoter region (Figure 5C). A clear band indicating the binding between FKHR protein and DBE oligonucleotides was detected, but ΔN-FKHR could not bind to DBE oligonucleotides. Moreover, the specific band disappeared by the addition of 100-fold excess of unlabelled wild-type, but not mutant, DBE oligonucleotides.

DISCUSSION

The present study revealed that the expression pattern of FOXO genes shows a tissue-specific and time-dependent change in response to starvation stress. Indeed, the gene expression of FKHR and FKHRL1 was remarkably and selectively induced in skeletal

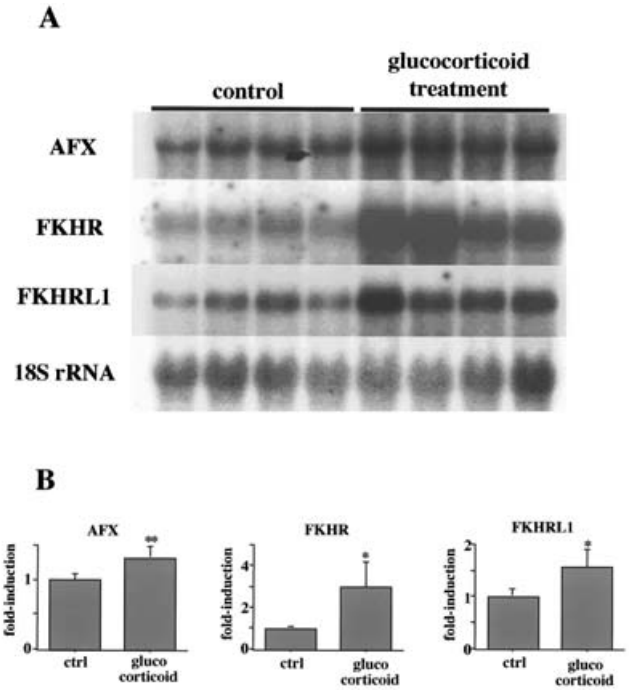

Figure 3 High induction of FKHR gene expression in skeletal muscle of glucocorticoid-treated mice

(**A**) and (**B**) show induction of FKHR gene expression by glucocorticoid treatment in gastrocnemius muscle. (**A**) Northern blot analysis was performed by using total RNAs from gastrocnemius muscle of mice after treatment with 1 mg of glucocorticoid or saline alone for 6 h. The intrinsic standard was 18 S rRNA. (**B**) Results in (**A**) were quantified and are means of fold-induction over the control (ctrl) ± S.E.M. ($n = 4$). $^{*}P < 0.05$.

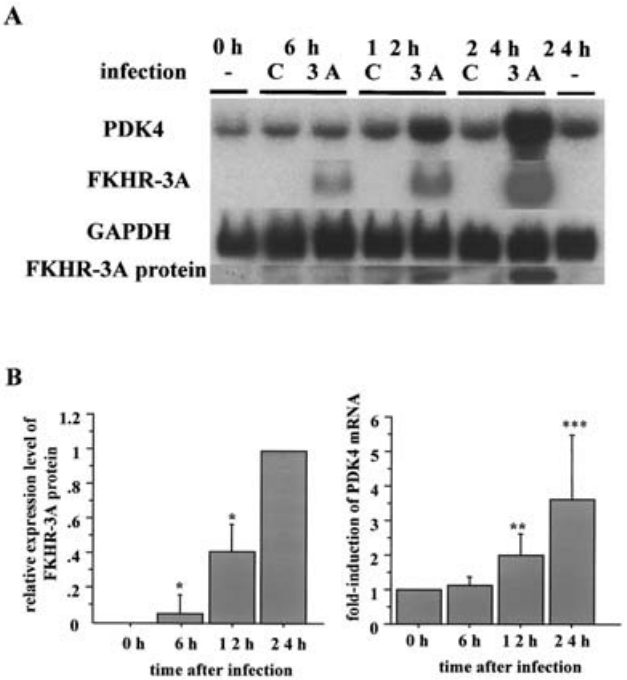

Figure 4 Up-regulation of PDK4 gene expression by FKHR in differentiated C2C12 cells

(**A**) Northern blot analysis was performed by using total RNAs from differentiated C2C12 cells for 3 days (D3) (= 0 h) or 4 days (D4) (= 24 h), and 6, 12 and 24 h after infection at D3 with both adenoviruses carrying a constitutively active FKHR (FKHR-3A) with myc-tag flanked by loxP and Cre recombinase or the latter alone using probes specific for PDK4 and FKHR. Exogenous FKHR-3A protein was detected by Western blot analysis using an antibody against the myc tag. The intrinsic standard was G3PDH. C, Cre alone; 3A, constitutively active FKHR and Cre. (**B**) Analysis of the expression level of exogenous FKHR-3A protein and induction level of PDK4 gene expression obtained in three independent experiments. Results are means ± S.E.M. ($n = 3$). $^{*}P < 0.0001$ compared with the 24 h group, $^{**}P < 0.02$ and $^{***}P < 0.0001$ compared with the 0 h group.

muscle during starvation, whereas the expression level of the AFX gene was routinely high and showed no remarkable changes by starvation in the tissue. There were no remarkable changes in the levels of FOXO genes expression in liver and kidney, with abundant expression of PEPCK and G6P during starvation [14,15] (Figure 1). The increased expression of FKHR gene during starvation may be ascribed in part to a high level of blood glucocorticoid [20], as suggested in the present study. It is also possible that increased plasma non-esterified fatty acids (NEFAs) have an effect on the gene expression of FKHR through peroxisome-proliferator-activated receptors (PPARs), because NEFA levels are known to be elevated during starvation and diabetes [21]. Besides these factors, oestrogen and gonadotropins are also known to regulate the expression of the FKHR gene in granulosa cells of developing follicles of ovary [14]. Therefore, it is suggested that the expression of FKHR gene is under the control of a variety of substrates produced and hormones secreted in response to physiological and pathological conditions in a specific tissue.

It is expected that low levels of blood insulin during starvation or diabetes lead to a low activity of Akt and an increase in the amount of unphosphorylated FKHR protein, which exerts transcriptional activity owing to localization into the nuclei [10,11]. The level of phospho-Ser-256 FKHR protein was actually increased by 1.3-fold in skeletal muscle by 24 h of starvation, probably because of phosphorylation by kinases other than Akt (Figure 2D). However, as a result of a 2.5-fold increase in the expression of FKHR protein during starvation, the amount of unphosphorylated FKHR protein would be much larger than during *ad libitum* feeding (Figure 2D). Taken together, it is conceivable that the change in the level of FKHR gene expression is more important than that of phosphorylation in the regulation of target gene expression, at least in skeletal muscle during starvation.

It is important to know which type of genes is induced by FKHR for our understanding of a role during starvation. The targets may comprise enzymes involved in glucose metabolism such as PDK4, and polyubiquitin and proteasome subunits, which contribute to protein degradation. Indeed, the expression of these genes is induced in skeletal muscle during starvation [15,22–24], and the expression of PDK4 gene is down-regulated by insulin [16,25]. PDK4 is a critical factor for maintaining the level of blood glucose during starvation. High levels of PDK4 protein would act to reduce the activity of pyruvate dehydrogenase, which catalyses the reaction from pyruvate to acetyl-CoA, and would decrease the consumption of 3-carbohydrates in skeletal muscle during starvation [17,26]. Recently, it was reported that PPARα has an important role in regulation of PDK4 gene expression in the tissues involved in energy metabolism [16,27–32]. PPARα is critical for the up-regulation of PDK4 gene expression in the liver and kidney during starvation, because of no induction of the gene in the starved tissues of PPARα-null mice [27–29]. However, enhanced PDK4 protein expression after starvation is retained in slow oxidative skeletal muscle and fast oxidative-glycotic skeletal muscle of PPARα-null mice [31,32]. These results suggest that there is a mechanism of regulation of PDK4 gene expression besides PPARα in skeletal muscle during starvation. The gene regulation by FKHR is a good candidate for

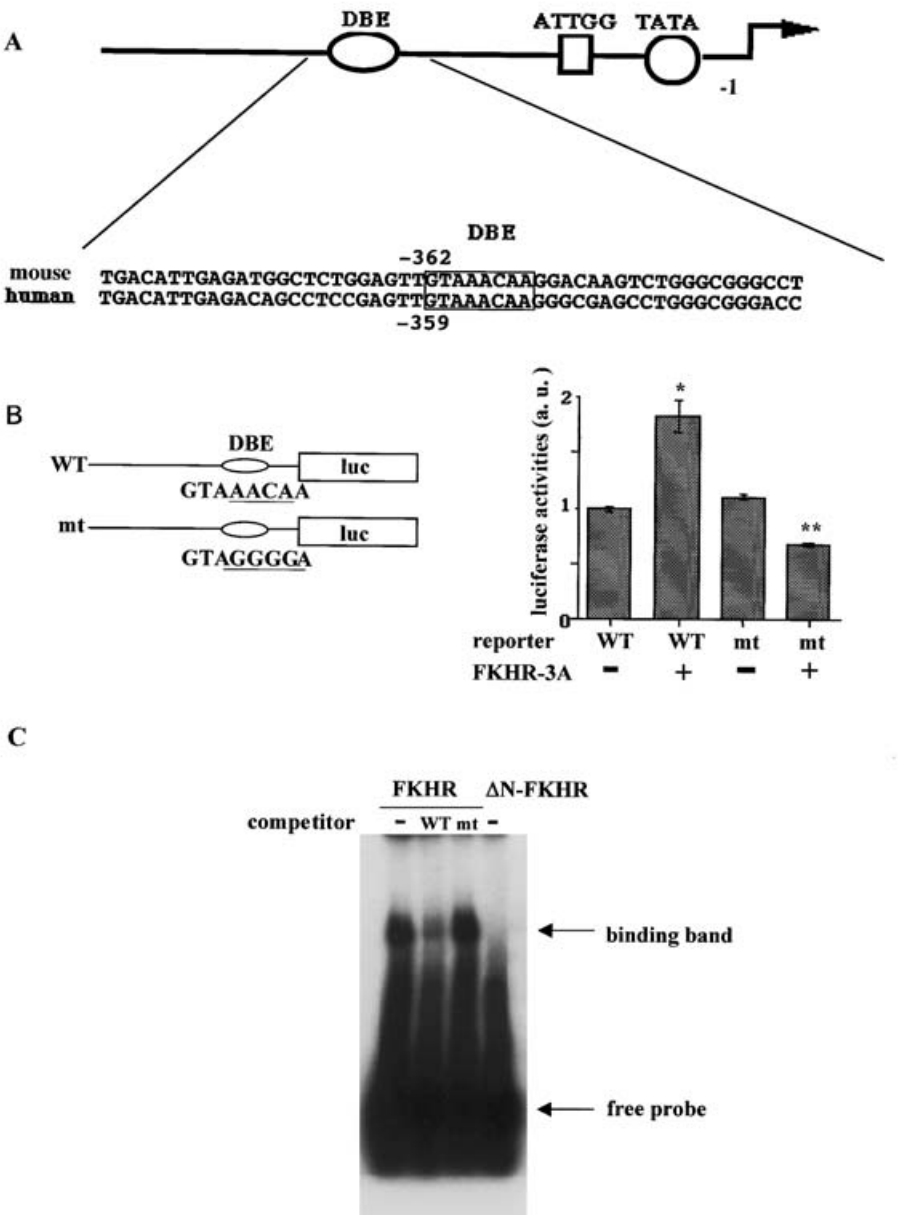

Figure 5 FKHR directly regulates PDK4 gene through DBE in the promoter region of PDK4 gene

(**A**) A DBE in the promoter region conserved between mouse and human PDK4 genes. (**B**) Reporter assays using C2C12 cells were performed with a reporter vector including a 972 bp promoter region of the mouse PDK4 gene (WT) fused to a luciferase gene or one with a 4 bp replacement (AACA → GGGG) in DBE in the promoter region (mt). Results are means ± S.E.M. ($n = 3$). *$P < 0.001$ and **$P < 0.005$, compared with the LacZ control vector. (**C**) GMSA was performed for wild-type (WT) DBE and mutant-type (mt) DBE oligonucleotides using Trx-fused FKHR protein (FKHR) and Trx-fused DNA-binding-domain-deficient FKHR protein (ΔN-FKHR).

a new mechanism as shown in the present study. However, FKHR alone may not be sufficient for the approx. 11-fold induction of PDK4 gene expression seen by 24 h of starvation, because overexpressed FKHR-3A protein in C2C12 cells was able to lead to only a 3.5-fold induction of the gene expression. Although the marked induction of PDK4 gene expression was coincident with the remarkable increase of FKHR protein at 24 h after food deprivation, the induction of PDK4 gene expression began to be observed at 6 h after food deprivation when the level of FKHR protein was not significantly increased. Thus it is conceivable that FKHR regulates PDK4 gene expression co-ordinately with other factors, such as PPARα. It remains to be determined whether or not enhanced expression of FKHR protein is also observed in other types of muscle fibres, and whether or not FKHR regulates PDK4 gene expression in concert with PPARα.

Most recently, it was reported that the gene expression of lipoprotein lipase, a rate-limiting enzyme in lipid usage in skeletal muscle, was induced by FKHR in skeletal muscle during starvation [33]. Taken together with our findings, this implies that FKHR acts as a switch for a shift toward the use of lipids from glucose as fuel substrate in skeletal muscle during starvation. Caloric restriction (CR) has many anti-aging effects on a variety of tissues [34]. For example, CR can reverse the metabolic shift toward the use of glucose from lipids observed in the aging heart [35]. To our knowledge, there are no reports showing the reversed shift in skeletal muscle under CR. Since we previously showed

that CR periodically increases the level of FKHR gene expression in skeletal muscle [36], CR may enhance the metabolic shift from glucose to fatty acid metabolism via FKHR also in skeletal muscle. These results suggest that FKHR plays an important role in the regulation of energy metabolism in skeletal muscle in response to changes of physiological and pathological conditions.

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