Endoplasmic Reticulum Stress-Induced Activation of Activating Transcription Factor 6 Decreases cAMP-Stimulated Hepatic Gluconeogenesis via Inhibition of CREB

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The expression of genes encoding key hepatic gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), is regulated at the transcriptional level by a network of transcription factors and cofactors, including cAMP response element-binding protein (CREB). It has been suggested that increased endoplasmic reticulum (ER) stress in the liver impairs hepatic glucose metabolism. However, the direct effect of ER stress on hepatic gluconeogenesis is still not clear. Here, we investigated whether ER stress influences hepatic gluconeogenesis and whether this process is mediated by activating transcription factor 6 (ATF6) through the inhibition of cAMP-mediated activation of CREB. A cAMP stimulant, forskolin, and 8-bromoadenosine-cAMP increased PEPCK and G6Pase mRNA expression in H4IIE rat hepatoma cells, and ER stress induced by tunicamycin or thapsigargin decreased the expression of these genes in forskolin or 8-bromoadenosine-cAMP-treated cells. In a transient transfection study, ATF6 inhibited the PEPCK and G6Pase promoters. Also, adenovirus-mediated overexpression of ATF6 in H4IIE cells decreased forskolin-stimulated PEPCK and G6Pase gene expression. Moreover, the inhibition of endogenous ATF6 expression by small interfering RNAs restored the ER stress-induced suppression of PEPCK and G6Pase gene expression. Transient transfection of ATF6 inhibited transactivation by CREB on the PEPCK and G6Pase promoters, and a gel shift assay showed that Ad-ATF6 inhibits forskolin-stimulated CREB DNA-binding activity. Finally, we found that expression of ATF6 decreased fasting-induced PEPCK, G6Pase mRNA expression, and blood glucose levels in mice. Taken together, these data extend our understanding of ER stress and the regulation of liver gluconeogenesis by ATF6. (*Endocrinology* 151: 561–568, 2010)

Glucose homeostasis is maintained by balancing glucose production in the liver with glucose use by peripheral tissues (1, 2). Under fasting conditions, hepatic gluconeogenesis is strongly stimulated by transcriptional activation of gluconeogenic genes by glucagon via the cAMP axis, but during feeding, this process is inhibited by insulin (3, 4). Hepatic gluconeogenesis is regulated mainly by the activities of key enzymes such as phosphoenolpyru-

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Abbreviations: ATF6, Activating transcription factor 6; 8-br-cAMP, 8-bromoadenosine-cAMP; CHOP, CCAAT/enhancer-binding protein homologous protein; CRE, cAMP response element; CREB, CRE binding protein; CRTC2, CREB regulated transcription coactivator 2; ER, endoplasmic reticulum; GFP, green fluorescent protein; G6Pase, glucose-6-phosphatase; IRE, interferon response element; MOI, multiplicity of infection; PEPCK, phosphoenolpyruvate carboxykinase; PERK, pancreatic ER kinase; PGC-1α, peroxisome proliferator-activated receptor γ-coactivator-1α; siRNA, small interfering RNA; Tg, thapsigargin; Tm, Tunicamycin; XBP, X-box binding protein.

vate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (5). The expression of the genes encoding these enzymes is strongly regulated at the transcriptional level by a network of transcription factors and cofactors, such as cAMP response element (CRE)-binding protein (CREB) (6), CREB regulated transcription coactivator 2 (CRTC2) (7), peroxisome proliferator-activated receptor γ-coactivator-1α (PGC-1α) (8), and small heterodimer partner (9). cAMP signaling leads to CREB phosphorylation at Ser133 (10). Phospho-CREB, bound to the CRE, then recruits the coactivators CBP and p300, which activate gene transcription through their intrinsic histone acetyltransferase activity and through recruitment of other coactivator molecules (11, 12).

Recently, numerous studies have shown the importance of endoplasmic reticulum (ER) stress in the pathogenesis of various liver diseases, including chronic viral hepatitis, insulin resistance, nonalcoholic fatty liver disease, ischemia-reperfusion injury, genetic disorders of protein misfolding, and alcoholic liver disease (13–15). However, the direct effects of ER stress on hepatic glucose metabolism have not been well established. The ER stress response involves the function of three molecular components: pancreatic ER kinase (PERK), interferon response element (IRE)-1/X-box binding protein (XBP)-1, and activating transcription factor 6 (ATF6) (16). Among these, ATF6 is a member of the ATF/CREB family (17). The mammalian ATF/CREB family represents a large group of basic-region leucine zipper transcription factors with rather diverse physiological functions (17). However, despite their different activities, ATF/CREB family members share a common ability to respond to environmental signals and maintain cellular homeostasis. For example, ATF2, ATF3, and ATF6 play roles in mediating stress responses: ATF2 and ATF3 have been implicated in transcriptional control of stress-response genes (18), whereas ATF6 is involved in regulating the expression of genes associated with ER stress and serum responses (19). Furthermore, CREB and ATF1 are involved in modulating transcription in response to intracellular cAMP concentrations, and ATF4 acts as a negative regulator of CRE-dependent transcription (17). The consensus binding site for ATF is TGACGT(C/A)(G/A) (20), a sequence identical to the CRE consensus (TGACGTCA) (21). However, no study has suggested that ATF6 plays a role in regulating CRE-dependent genes. Moreover, the mechanism of transcriptional regulation of PEPCK and G6Pase by ATF6 has not been fully defined.

In this study, we examined whether ER stress influences hepatic gluconeogenesis and whether this process is mediated by ATF6 inhibition of cAMP-mediated activation of CREB activity.

Materials and Methods

Chemicals

Tunicamycin (Tm; Sigma, St. Louis, MO), thapsigargin (Tg; Sigma), 8-bromoadenosine-cAMP (8-br-cAMP; Sigma), and the cAMP stimulant forskolin (Sigma) were purchased and dissolved in the recommended solvents.

Cell culture

The H4IIE rat hepatoma cell line and HepG2 human hepatoma cell line were cultured in 5% CO_2/96% air at 37 C in MEM (Life Technologies, Inc.-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics. After reaching approximately 80% confluence, cells were serum-starved for 24 h in medium containing 0.5% fetal bovine serum and then treated as indicated in the text.

Generation of recombinant adenovirus

The cDNA encoding a constitutively active form of ATF6 (amino acids 1-373, 50-kDa, cytosolic N-terminal portion of ATF6) was inserted into the pAdTrack-CMV shuttle vector. The vector construct was then electroporated into BJ5138 cells, and a recombinant vector was generated using the AdEasy adenoviral vector system. The recombinant viruses were amplified in HEK-293 cells and purified by CsCl (Sigma) gradient centrifugation. Viral preparations were collected and desalted, and titers were determined using Adeno-X rapid titer (BD Bioscience, San Jose, CA), according to the manufacturer's instructions. The efficiency of adenoviral infection was assessed using a recombinant adenovirus encoding ATF6 fused to green fluorescent protein (GFP) (data not shown).

Construction of a small interfering RNA (siRNA) for ATF6

The ATF6 siRNA was chemically synthesized by Bioneer (Seoul, Korea), deprotected, and annealed. Transfections were performed according to the manufacturer's instructions. Briefly, H4IIE cells were transfected with the ATF6 siRNA oligonucleotide (100 nM) using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Cells were transfected for 4 h, serum-starved for 16 h, and treated with Tm (2 μg/ml) and forskolin (30 μM) for an additional 12 h. Cells were harvested, and the total RNA was isolated for Northern blot analysis. The sequences of the ATF6 and nonspecific control siRNAs were as follows: rat ATF6 siRNA, CCA UUG UGU UAC CAG CAA U tt (sense); nonspecific control siRNA, GGA GUA CGC AUA CCU GAA AGG tt (sense). The effects of ATF6 siRNA on the expression of ATF6 mRNA were measured by RT-PCR.

Northern blot analysis

H4IIE cells were treated with Tm, Tg, forskolin (30 μM), or 8-br-cAMP (500 μM) and subsequently infected with adenoviral vectors expressing ATF6. Cells were harvested at the indicated times, total RNA was isolated using Trizol reagent (Invitrogen), according to the manufacturer's instructions, and 20 μg of total RNA from each sample was used. The probes for PEPCK, G6Pase, and ATF6 were labeled with [α-^{32}P]dCTP using a ran-

dom-primer DNA-labeling system (Amersham Biosciences, Little Chalfont, UK).

Western blot analysis

H4IIE cells were treated with Tm and Tg for various times and then harvested in lysis buffer [50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Nonidet P-40, 0.25% Na-dexoycholate] containing proteinase inhibitors. The proteins were resolved by SDS-PAGE and then transferred electrophoretically to a polyvinyl difluoride membrane (Millipore, Bedford, MA). The membrane was blocked by incubation in blocking buffer, incubated with anti-ATF6 antibody (Labprontier, Seoul, Korea), anti-ATF4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-CCAAT/enhancer-binding protein homologous protein (CHOP) antibody (Santa Cruz), anti-FoxO1 antibody (Cell Signaling, Beverly, MA), and antiphosphorylated (p)-FoxO1 antibody (Cell Signaling), and was then developed using an ECL Western blot detection kit (Amersham Biosciences). The membrane was reblotted with antiactin antibody to verify equal loading of protein in each lane. Densitometry was used to quantitate the results, using the digitalized scientific software program UN-SCAN-IT (Skik Scientific Corp., Orem, UT).

Analysis of XBP-1 mRNA splicing

Total RNA was obtained from H4IIE cells using Trizol reagent (Invitrogen). cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas, Hanover, MD) and 2 μg of total RNA, according to the manufacturer's instructions. PCR was carried out under the following conditions using *Taq* polymerase (Takara, Tokyo, Japan): 92 C for 3 min for 40 cycles, 92 C for 45 sec, 52 C for 45 sec, and 72 C for 45 sec. The following primers were used: XBP-1, 5′-AAA CAG AGT AGC AGC GCA GAC TGC-3′ (forward) and 5′-GGA TCT CTA AAA CTA GAG GCT TGG TG-3′ (reverse); and β-actin, 5′-GGC ATC GTC ACC AAC TGG GAC-3′ (forward), and 5′-CGA TTT CCC GCT CCG TGG-3′ (reverse). The purified PCR products were digested by PstI for 5 h at 37 C, then separated by 2% agarose gel electrophoresis.

In vitro transient transfection and gene reporter assays

HepG2 cells were plated at a density of 7×10^4 cell/well in a 12-well plate and cultured for 1 d in culture medium. Cells were transiently transfected with the indicated promoter constructs (200 ng/well) and other cDNAs using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were cotransfected with a plasmid encoding β-galactosidase as an internal control. Cells were transfected for 4 h, serum-starved for 16 h, and treated with forskolin (30 μM) for an additional 8 h. Cells were harvested and assayed for luciferase and β-galactosidase activity. Twenty microliters of cell lysate containing 15 μg of protein were analyzed using the luciferase assay system, according to the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was measured using a SIRUS luminometer (Berthold, Pforzheim, Germany) and was normalized to β-galactosidase activity. Reporter plasmids encoding the human G6Pase promoter (−1227/+57) and the rat PEPCK promoter (−2000/+73) were generously provided by D. Schmoll (22) and R. W. Hanson (23), respectively.

EMSA

Nuclear extracts were prepared from HepG2 cells using the NucBuster protein extraction kit (Calbiochem, La Jolla, CA), according to the manufacturer's instructions. Nuclear extracts (6 μg) were incubated at room temperature with 60,000 cpm of a ^{32}P-labeled double-stranded oligonucleotide encoding the PEPCK CRE1 sequence (promoter positions −99 to −76) 5′-CCGGCCCCTTACGTCAGAGGCG-3′ as a probe (24, 25). Competition experiments were performed with 10× unlabeled oligonucleotide sequences for PEPCK CRE1. Supershift experiments were performed by incubating the binding reaction with 0.4 μg of supershifting antibody (anti-CREB antibody; Santa Cruz). Samples were separated in 4% acrylamide, 0.5× TBE [0.045 M Tris, 0.045 M boric acid, 1.0 mM EDTA (pH 8.0)] gels run at 150 V constant voltage (26).

Animals

In vivo experiments were conducted using 8- to 9-wk-old male C57BL/6 mice (Samtako, Korea). All animal procedures were carried out in accordance with institutional guidelines for animal research. ER stress was induced in C57Bl/6 mouse livers by Tm (0.1 mg) injection into the tail vein. Mice were fasted for 16 h and killed for liver collection. Exogenous overexpression of ATF6 or GFP was induced in the livers of C57Bl/6 mice via injection of 1×10^9 pfu/200 μl of adenovirus into the tail vein. After 3 d, mice were fasted for 16 h and killed for blood and liver collection. Nine mice per group were used in this experiment. Blood glucose levels were measured using glucose reagent strips and a glucometer (Abbott, Bedford, MA).

Statistical analysis

Data are the means ± SE. ANOVA was used to determine significant differences in multiple comparisons and was performed using the Duncan test. $P < 0.05$ was considered statistically significant. All experiments were performed at least three times.

Results

Chemical-induced ER stress decreases PEPCK and G6Pase gene expression

To determine whether experimental induction of ER stress regulates gluconeogenic gene expression, we used Northern blot analysis to examine the effects of Tm and Tg on PEPCK and G6Pase mRNA expression. Tm and Tg each decreased PEPCK mRNA expression with 6–12 h of exposure (Fig. 1A). The level of G6Pase increased slightly after 3 h of exposure to Tm and Tg, but it then decreased upon longer exposure (6–12 h) (Fig. 1A). The induction of ER stress was confirmed by determining the expression of various genes implicated in the unfolded protein response: The activated form of ATF6 (50-kDa, cytosolic N-terminal portion of ATF6), ATF4, and CHOP were expressed at low levels under basal conditions, and exposure to Tm and Tg increased their expression in a time-dependent manner (Fig. 1B). Splicing of XBP-1 was induced after 1 h of Tm and Tg exposure (Fig. 1C).

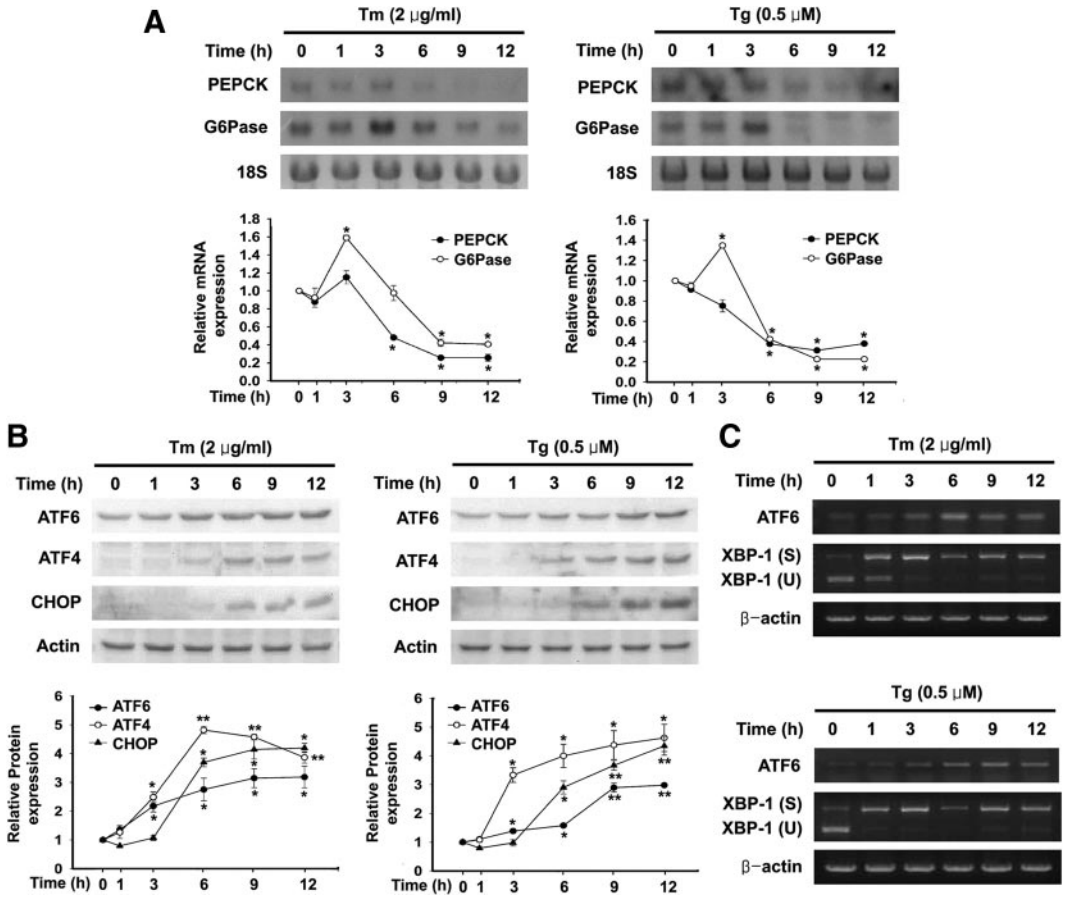

FIG. 1. ER stress inhibits PEPCK and G6Pase gene expression. A, Representative Northern blot analysis of PEPCK and G6Pase mRNA expression in H4IIE cells treated with 2 μg/ml Tm and 0.5 μM Tg for the time periods shown. 18S rRNA levels were analyzed as an internal control. Data in the *line graph* are the means ± SE of three independent measurements. *, $P < 0.01$, compared with 0 h. B, Representative Western blot analysis of ATF6 (50 kDa, active form of ATF6), ATF4, and CHOP in the presence of Tm and Tg for the time periods shown. Data in the *line graph* are the means ± SE of three independent measurements. *, $P < 0.05$; **, $P < 0.01$ compared with 0 h. C, Representative RT-PCR analysis of ATF6 and XBP-1 splicing in the presence of Tm and Tg for various time periods, with mRNA levels assessed at each time point. S, spliced form; U, unspliced form. β-Actin mRNA levels were analyzed as an internal control.

To determine whether ER stress-induced inhibition of hepatic gluconeogenesis involves a cAMP signaling pathway, we examined the effects of ER stress on the expression of PEPCK and G6Pase in 8-br-AMP- and forskolin-stimulated cells, because forskolin is a known activator of cAMP function. As shown in Fig. 2, H4IIE cells treated with forskolin or 8-br-cAMP for 12 h resulted in an increase in PEPCK and G6Pase mRNA expression, but cotreatment with Tm or Tg repressed the forskolin or 8-br-cAMP-stimulated PEPCK and G6Pase gene expression in a dose-dependent manner.

ER stress-induced inhibition of PEPCK and G6Pase expression is mediated by ATF6

We next examined which of the three different signaling arms of the ER stress response (PERK, IRE1, or ATF6) mediates the inhibition of gluconeogenic gene expression. As shown in Fig. 3A, transient expression of the active form of ATF6 in HepG2 cells significantly inhibited forskolin-stimulated PEPCK and G6Pase promoter activity. In contrast, although the expression of ATF4, a key component of the PERK pathway, tended to decrease forskolin-stimulated G6Pase promoter activity, the effect of ATF4 was much less than that of ATF6. Moreover, ATF4 had no effect on forskolin-stimulated PEPCK promoter activity. Quite unexpectedly, the expression of the spliced form of XBP-1, which functions downstream of IRE1, further increased the forskolin-stimulated PEPCK and G6Pase promoter activity. The transfection efficiency was determined by Western blot analysis (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

Based on the results of the transient transfection assay, we were interested in determining the physiological role of ATF6 in gluconeogenic gene expression in H4IIE cells. To determine the effect of ATF6 on the expression of the PEPCK and G6Pase genes, H4IIE cells were infected with an adenovirus encoding a constitutively active form of ATF6 (Ad-ATF6) at a multiplicity of infection (MOI) of 30

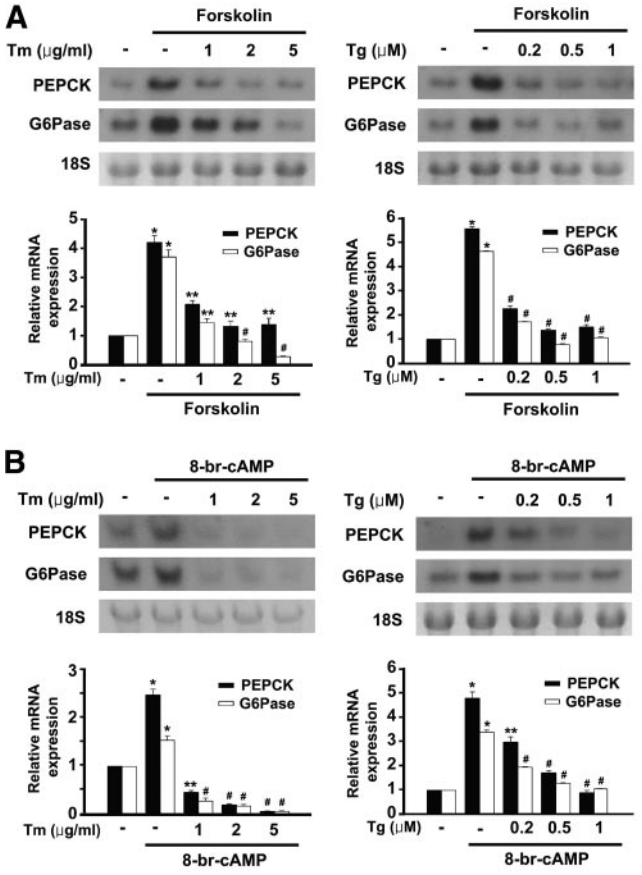

FIG. 2. ER stress inhibits forskolin- or 8-br-cAMP-stimulated PEPCK and G6Pase gene expression. A, Representative Northern blot analysis showing the effect of ER stress on forskolin-stimulated PEPCK and G6Pase mRNA expression. H4IIE cells were incubated with forskolin (30 μM) for 12 h and with Tm and Tg at various doses for 12 h. 18S rRNA levels were analyzed as an internal control. Data in the *bar graph* are the means ± SE of three independent measurements. *, $P < 0.001$ compared with control; **, $P < 0.01$; #, $P < 0.001$ compared with forskolin alone. B, Representative Northern blot analysis showing the effect of ER stress on 8-br-cAMP-stimulated PEPCK and G6Pase mRNA expression. H4IIE cells were incubated with 8-br-cAMP (500 μM), Tm (various doses), and Tg (various doses) for 12 h. The 18S rRNA levels were analyzed as an internal control. Data in the *bar graph* are the means ± SE of three independent measurements. *, $P < 0.01$ compared with control; **, $P < 0.05$; #, $P < 0.01$ compared with 8-br-cAMP alone.

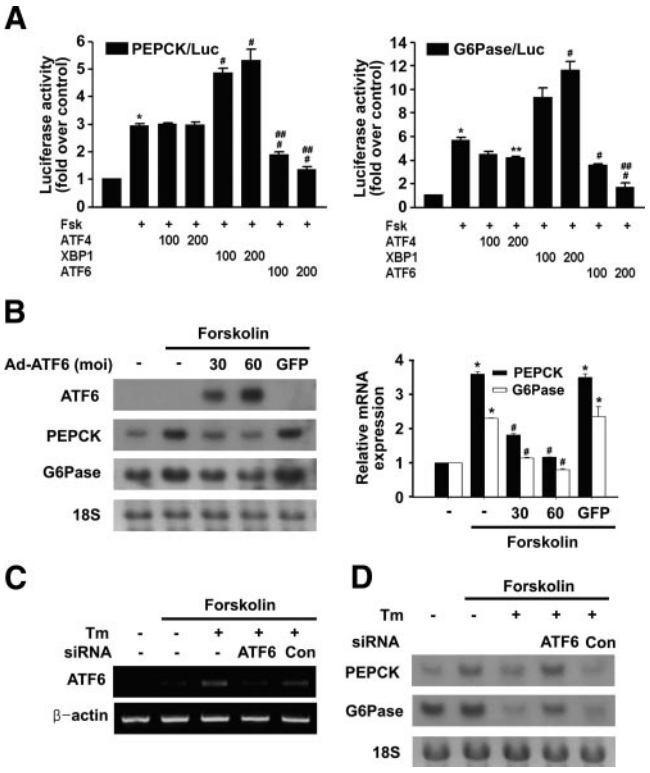

FIG. 3. ATF6 inhibits forskolin-stimulated PEPCK and G6Pase expression. Panel A, Effect of ATF4, the spliced form of XBP-1 and the active from of ATF6 on forskolin-stimulated PEPCK and G6Pase promoter activity. HepG2 cells were cotransfected with the PEPCK and G6Pase promoters (200 ng/well) and the indicated amounts of an expression vector for ATF4, XBP-1, or ATF6. Cells were then serum-starved for 16 h and treated with forskolin (Fsk) (30 μM) for an additional 8 h. Luciferase (luc) activity was normalized to β-galactosidase activity as an internal control. Data represent the means ± SE of three independent measurements. *, $P < 0.01$ compared with control; **, $P < 0.05$; #, $P < 0.01$ compared with forskolin alone; ##, $P < 0.05$ compared with forskolin/ATF4. Panel B, Representative Northern blot analysis showing the effect of overexpression of ATF6 on forskolin-stimulated PEPCK and G6Pase mRNA expression. H4IIE cells were infected with the indicated doses (MOI) of Ad-ATF6 or Ad-GFP for 24 h and treated with forskolin (30 μM). 18S rRNA levels were analyzed as an internal control. Data in the *bar graph* are the means ± SE of three independent measurements. *, $P < 0.01$ compared with control; #, $P < 0.01$ compared with forskolin alone. Panel C, H4IIE cells were transfected with 100 nM siRNA-ATF6 or control siRNA (Con) and then treated with forskolin and Tm. RNA levels were normalized using β-actin. Panel D, Representative Northern blot analysis of the effect of siRNA-ATF6 on the Tm-decreased forskolin-stimulated PEPCK and G6Pase gene expression. 18S rRNA levels were analyzed as an internal control.

or 60 after 12 h of pretreatment with forskolin. Consistent with the effect of ATF6 on PEPCK and G6Pase promoter activity, Ad-ATF6 significantly repressed forskolin-stimulated PEPCK and G6Pase gene expression in a dose-dependent manner (Fig. 3B). To further confirm whether suppression of PEPCK and G6Pase gene expression was mediated by ATF6, we down-regulated endogenous ATF6 expression by transfecting H4IIE cells with siRNA-ATF6 and incubated the cells in Tm-containing media. The siRNA-ATF6 significantly inhibited Tm-induced ATF6 expression (Fig. 3C) and restored the Tm-induced suppression of PEPCK and G6Pase gene expression (Fig. 3D).

ATF6 decreases CREB DNA-binding activity

Because the transcriptional activity of gluconeogenic gene expression is primarily regulated by the transcription factor CREB (27), we examined whether ATF6 inhibits the transactivation activity of CREB on the PEPCK and G6Pase promoters. Transient expression of CREB slightly increased PEPCK and G6Pase promoter activity, and in the presence of forskolin, CREB markedly increased PEPCK and G6Pase promoter activity (Fig. 4A). ATF6 repressed the CREB-stimulated PEPCK

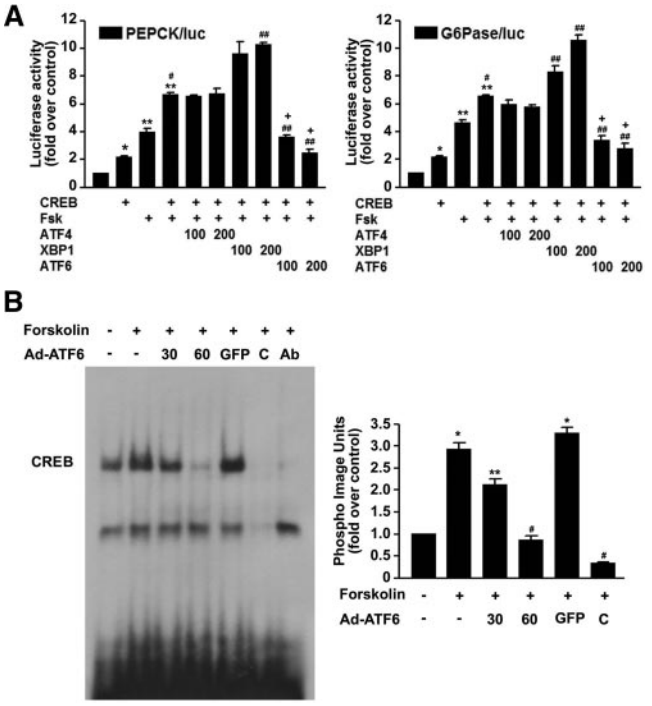

FIG. 4. ATF6 inhibits CREB DNA-binding activity. A, HepG2 cells were cotransfected with 200 ng each of the PEPCK and G6Pase reporter genes, CREB, and indicated amounts of an expression vector for ATF4, XBP-1, and ATF6. Cells were then serum-starved for 16 h and treated with forskolin (Fsk; 30 μM) for an additional 8 h. Luciferase (luc) activity was normalized to β-galactosidase activity as an internal control. Data represent the means ± SE of three independent measurements. *, $P < 0.05$; **, $P < 0.01$ compared with control; #, $P < 0.01$ compared with CREB or forskolin alone; ##, $P < 0.01$ compared with CREB/forskolin; +, $P < 0.01$ compared with CREB/forskolin/ATF4. B, The effect of ATF6 on forskolin-stimulated CREB DNA-binding activity. HepG2 cells were infected with the indicated doses (MOI) of Ad-ATF6 or Ad-GFP for 24 h and treated with forskolin (30 μM) for 3 h. Competition experiments (C) were performed with 10× unlabeled oligonucleotide sequences for CRE. Supershift experiments (Ab) were performed by incubating the binding reaction with anti-CREB antibody. Data represent the means ± SE of three independent measurements. *, $P < 0.01$ compared with control; **, $P < 0.05$; #, $P < 0.01$ compared with forskolin alone.

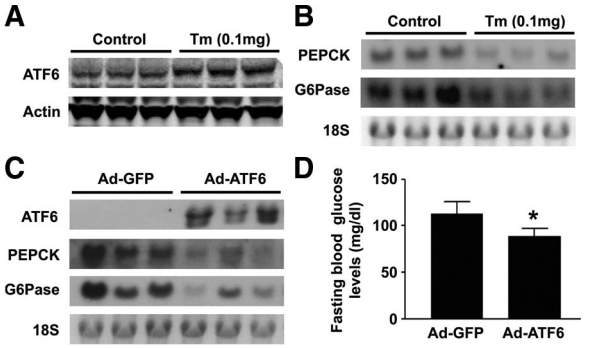

FIG. 5. ATF6 overexpression reduced fasting blood glucose levels, PEPCK, and G6Pase gene expression in C57BL/6 mice. A, Representative Western blot analysis of ATF6 (50 kDa, active form) expression in C57BL/6 mouse livers after tail vein injection with Tm (0.1 mg). Protein was extracted from liver tissues following a 16-h fast, and was analyzed by Western blot analysis. B, Representative Northern blot analysis of PEPCK and G6Pase mRNA expression in C57BL/6 mouse livers after tail vein injection with Tm (0.1 mg). Total RNA was extracted from liver tissues after a 16-h fast and was analyzed by Northern blot analysis. C, Representative Northern blot analysis of PEPCK and G6Pase mRNA expression in the livers of C57BL/6 mice after tail vein injection with Ad-ATF6 or Ad-GFP for 3 d. Total RNA was extracted from liver tissues in the 16-h fasting condition and was analyzed via Northern blot analysis. D, Fasting blood glucose levels of C57BL/6 mice were measured after tail vein injection with Ad-GFP or Ad-ATF6. Data represent the means ± SE of three independent measurements (n = 9 in each group). *, $P < 0.05$ compared with Ad-GFP.

and G6Pase promoter activity (Fig. 4A). In contrast, expression of ATF4 tended to decrease the CREB-stimulated G6Pase promoter activity, but had no effect on CREB-stimulated PEPCK promoter activity (Fig. 4A). The expression of the spliced form of XBP-1 additionally increased the CREB-stimulated PEPCK and G6Pase promoter activity (Fig. 4A). These findings prompted us to investigate whether ATF6 inhibited the ability of CREB to bind to the CRE region. To test whether ATF6 reduces CREB's DNA-binding activity, gel shift analyses were conducted with the CRE oligonucleotide. CREB's DNA-binding activity was stimulated by forskolin, and cells infected with Ad-ATF6 decreased this forskolin-stimulated CREB DNA-binding activity in a dose-dependent manner (Fig. 4B). These results suggest that ATF6-induced inhibition of gluconeogenic gene expression is mediated by suppression of CREB activity on the PEPCK and G6Pase promoters.

Overexpression of ATF6 decreases blood glucose levels and expression of the PEPCK and G6Pase genes in the livers of C57BL/6 mice

We next wanted to determine whether ER stress induction and ATF6 influence blood glucose levels and hepatic gluconeogenic gene expression in a CREB-stimulated condition *in vivo*. Hepatic glucose production under fasting conditions (see *Materials and Methods*) was analyzed in the livers of C57BL/6 mice treated with Tm or infected with ATF-encoding adenoviruses by tail vein injection. Treatment with Tm induced ATF6 expression *in vivo* (Fig. 5A). Tm treatment and adenovirus-mediated ATF6 overexpression decreased fasting-induced hepatic PEPCK and G6Pase gene expression (Fig. 5, B and C). Moreover, blood glucose levels in mice from the fasting condition were significantly decreased after overexpression of ATF6 compared with those in the livers of Ad-GFP-infected mice (Fig. 5D). Additionally, we checked whether ATF6 influenced PGC-1α expression and FoxO1 phosphorylation, which regulate gluconeogenic gene expression. PGC-1α expression and FoxO1 phosphorylation were not changed by ATF6, suggesting that the ATF6-mediated inhibition of gluconeogenic gene expression is not related to PGC-1 and FoxO1 (supplemental Fig. S2).

Discussion

We have shown that chemical-induced ER stress decreases PEPCK and G6Pase gene expression and that adenovirus-mediated overexpression of ATF6 decreases hepatic gluconeogenic gene expression and blood glucose levels both *in vivo* and *in vitro*. Transient transfection and gel shift assays suggest that ATF6 inhibits PEPCK and G6Pase gene expression via repression of CREB activity.

The ER stress response results in obesity, the deterioration of insulin action, and the development of type 2 diabetes (28). It has been suggested that increased ER stress in the liver induces hepatic insulin resistance and impairs hepatic glucose metabolism (29, 30). However, the direct effect of ER stress on hepatic gluconeogensis is still not clear. A recent study showed that experimental induction of ER stress by treatment with Tm or Tg for 2 or 4 h increases G6Pase expression via increased transcription (31). More recently, Gonzales *et al.* (32) demonstrated that short-term, chemically induced ER stress in the liver increases glucose production via mechanisms that involve increased glycogenolysis and liver phosphorylase activity. Consistent with a previous study by Wang *et al.* (31), we showed that short-term induction of ER stress increased G6Pase mRNA expression. However, long-term exposure (6–12 h) to Tm and to Tg decreased the expression of G6Pase mRNA. In addition, PEPCK mRNA expression gradually decreased after exposure to Tm and Tg without an initial increase, as was seen for G6Pase mRNA expression. Furthermore, we showed that treatment of Tm and Tg repressed forskolin- or 8-br-cAMP-stimulated PEPCK and G6Pase promoter activity and mRNA expression. Collectively, these results raise the possibility that, although the early phase of ER stress increases hepatic gluconeogenesis, consequent unfolded protein response suppresses hepatic gluconeogenesis in response to long-term induction of ER stress.

The ER stress response involves the functioning of three molecular components: PERK, IRE-1/XBP-1, and ATF-6. Among these, we found that ER stress-induced suppression of PEPCK and G6Pase promoter activity was mediated by the activation of ATF6 and that the other ER stress response pathways, such as those involving IRE1-XBP1 and PERK-eIF2α-ATF4, were less likely to be involved. Adenovirus-mediated ATF6 overexpression in H4IIE cells significantly repressed forskolin-stimulated PEPCK and G6Pase gene expression, and the inhibition of endogenous AFT6 expression by siRNA restored the ER stress-induced suppression of PEPCK and G6Pase gene expression. Moreover, fasting-induced PEPCK and G6Pase gene expression and blood glucose levels were decreased in C57BL/6 mice infected with adenoviruses encoding ATF6. These results suggest that ER stress-induced activation of ATF6 decreases PEPCK and G6Pase gene expression.

Hepatic gluconeogenesis is strongly stimulated during fasting by augmenting glucagon-dependent activation of CREB-mediated gene induction (27). The binding of CREB to the CRE, TGACGTCA, which is present in the regulatory sequences of many eukaryotic genes, results in stimulation or repression of the target genes (33, 34). This consensus binding site for CRE is identical to the ATF consensus (TGACGT[C/A][G/A]) (20), suggesting that ATF6 may influence CRE binding activity on the PEPCK and G6Pase promoters. Thus, we investigated whether ATF6 represses CREB-mediated transcription. Transient transfection and gel shift assays showed that ATF6 repressed CREB-stimulated PEPCK and G6Pase promoter activities and the CREB DNA-binding activity without the binding of ATF6 to the CRE. Additional studies are needed to elucidate the mechanism by which ATF6 inhibits the binding of CREB to the CRE. While we were preparing this manuscript, Wang *et al.* (35) reported that ER stress induced the dephosphorylation and nuclear entry of the CREB coactivator CRTC2, which in turn promoted the expression of ER quality control genes through an association with ATF6. Furthermore, ATF6 also reduced PEPCK and G6Pase gene expression by disrupting the CREB-CRTC2 interaction. Thus, it is possible that the inhibition of CREB coactivators such as CRTC2 may influence the CREB DNA-binding activity, although the mechanism of repression of CREB DNA-binding by ATF6 is currently not clear. Collectively, these results suggest that ATF6-induced inhibition of gluconeogenic gene expression is mediated by the suppression of CREB activity on the PEPCK and G6Pase promoters.

In conclusion, ER stress-induced activation of ATF6 decreases PEPCK and G6Pase gene expression as well as blood glucose levels. These data extend our understanding of ER stress and ATF6 regulation of liver gluconeogenesis.

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