Central Activating Transcription Factor 4 (ATF4) Regulates Hepatic Insulin Resistance in Mice via S6K1 Signaling and the Vagus Nerve

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Recent studies have revealed that the central nervous system, particularly the hypothalamus, is critical for regulating insulin sensitivity in peripheral tissues. The aim of our current study is to investigate the possible involvement of hypothalamic activating transcription factor 4 (ATF4) in the regulation of insulin sensitivity in the liver. Here, we show that overexpression of ATF4 in the hypothalamus resulting from intracerebroventricular injection of adenovirus expressing ATF4 induces hepatic insulin resistance in mice and that inhibition of hypothalamic ATF4 by intracerebroventricular adenovirus expressing a dominant-negative ATF4 variant has the opposite effect. We also show that hypothalamic ATF4-induced insulin resistance is significantly blocked by selective hepatic vagotomy or by inhibiting activity of the mammalian target of rapamycin (mTOR) downstream target S6K1. Finally, we show that inhibition of hypothalamic ATF4 reverses hepatic insulin resistance induced by acute brain endoplasmic reticulum (ER) stress. Taken together, our study describes a novel central pathway regulating hepatic insulin sensitivity that is mediated by hypothalamic ATF4/mTOR/S6K1 signaling and the vagus nerve and demonstrates an important role for hypothalamic ATF4 in brain ER stress–induced hepatic insulin resistance. These results may lead to the identification of novel therapeutic targets for treating insulin resistance and associated metabolic diseases. *Diabetes* 62:2230–2239, 2013

T he incidence of type 2 diabetes has increased tremendously in the world over the last 50 years in parallel with obesity, for which insulin resistance is a common feature. Many previous studies have investigated insulin signaling in the peripheral tissues, including liver, muscle, and adipose tissue, to uncover the causes of insulin resistance (1). Recent studies, however, have produced evidence for a role of the central nervous system (CNS), particularly the hypothalamus, in regulating insulin sensitivity in peripheral tissues (2–4). Signaling pathways in the hypothalamus directed from both S6K1, a downstream effector for the kinase mammalian target of rapamycin (mTOR), and the transcription factor NF-κB have been shown to contribute to insulin resistance in the liver (3,5). It has also been shown that the hepatic branch of the vagus nerve is involved in the hypothalamic control of hepatic glucose metabolism (4,6,7).

The transcription factor activating transcription factor 4 (ATF4) belongs to the cAMP-responsive element–binding protein family, characterized by the presence of a leucine zipper dimerization domain and a basic amino acid–rich DNA binding domain (8). Previous studies have shown that knocking out *ATF4* gene expression in mice results in anemia (9) and abnormal development of the eye (10). Recent studies have also implicated ATF4 in the regulation of energy homeostasis and glucose metabolism (11–14). For example, *ATF4* knockout (KO) mice exhibit decreased fat mass and increased energy expenditure due to increased thermogenesis (11,12). These mice also show enhanced insulin sensitivity and resistance to high-fat diet (HFD) or high-carbohydrate diet–induced hyperglycemia (11,13,14). Recently, a study using tissue-specific KO mice demonstrated that ATF4 regulates glucose metabolism in mice through its expression in osteoblasts (13). Although *ATF4* mRNA is ubiquitously expressed (8), the role of ATF4 in the regulation of insulin sensitivity in other tissues remains largely unknown.

Building on research demonstrating the importance of the hypothalamus in the regulation of peripheral glucose metabolism, the current study investigated whether hypothalamic ATF4 plays a role in the regulation of peripheral insulin sensitivity. Because brain endoplasmic reticulum (ER) stress is recognized as one of the primary causes for hepatic insulin resistance (3,15), we also investigated whether ATF4, as an ER stress–responsive target (16), is a central mediator for this regulation. Our results demonstrate that hypothalamic ATF4 plays an important role in regulating hepatic insulin sensitivity and is a key regulator mediating brain ER stress–induced insulin resistance.

RESEARCH DESIGN AND METHODS

Animals and treatment. Male C57BL/6J mice were obtained from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). Eight- to ten-week-old mice were maintained on a 12-h light-dark cycle at 25°C and provided with free access to commercial rodent chow and tap water prior to the experiments. At the end of experiments, animals were killed by CO_2 inhalation. Tissues were isolated, snap-frozen, and stored at $-80°C$ for future analysis. Normally, the same set of mice was used for measuring fed blood glucose levels prior to fasting, performance of insulin tolerance test (ITT), and examination of levels of hypothalamic proteins of interest and insulin signaling in the liver 7 days post adenoviral injection. Another set of mice was used for measuring levels of fasting blood glucose and serum insulin, performance of glucose tolerance test (GTT), and examination of RNA levels for proteins of interest in the hypothalamus 7 days post adenoviral injection. These experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences (INS), Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS).

Generation of recombinant adenoviruses. The ATF4 plasmid (17) and the plasmid encoding the dominant-negative mutant of ATF4 (DN-ATF4) (18) were provided by Dr. Zaiqing Yang (Huazhong Agricultural University, Wuhan, China) and Dr. Jawed Alam (Louisiana State University Health Sciences Center, New Orleans, LA), respectively. The recombinant adenoviruses used for expression of ATF4 (Ad-ATF4) and DN-ATF4 (Ad-DN-ATF4) were generated using

the AdEasy Adenoviral Vector System (Qbiogene) according to the manufacturer's instructions. Adenoviruses with either scrambled sequence (Ad-scramble) or adenovirus expressing small hairpin RNA directed against the coding region of S6K1 (Ad-shS6K1) were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The sequence designed for the knockdown of S6K1 is 5′-CACCGGGAGTTGGACCATATGAACTCGAAAGTTCATATGGTCCAACTCCC-3′.

Intracerebroventricular administration experiments. Intracerebroventricular administration experiments were conducted as previously described (3). An amount of 1 μL 4 × 10^8 pfu/mouse of Ad-ATF4, Ad-DN-ATF4, Ad-shS6K1, or control adenovirus was injected into the third ventricle (at the midline coordinates of 1.8 mm posterior to the bregma and 5.0 mm below the bregma) using a microsyringe. A 7-day postadenoviral injection protocol was taken based on previous reports (3,19) and our preliminary experiments showing that peak virus expression in the hypothalamus was found between 5 and 7 days, which was back toward baseline at 14 and 21 days. For thapsigargin (TG) administration experiments, mice were intracerebroventricularly injected with Ad-DN-ATF4 or Ad-GFP and implanted with cannula and then allowed to recover for 3 days. After recovery, mice were injected with 2 μL of 0.5 μg/μL TG (Sigma-Aldrich, St. Louis, MO; dissolved in artificial cerebrospinal fluid containing 10% DMSO) or control vehicle once daily for three consecutive days, as previously described (3).

Selective hepatic vagotomy. Hepatic branch vagotomy or sham surgery (isolation of the nerve without resection) was performed in mice as previously described (4). These mice were then intracerebroventricularly injected with Ad-ATF4 or Ad-GFP.

Primary hypothalamic neuron isolation and treatments. Primary cultures of hypothalamic neurons were prepared and cultured as previously described (20). On day 10, primary cultured neurons were transfected with plasmids encoding ATF4, DN-ATF4, or control vector using Lipofectamine 2000 Transfection Reagent (Life Technologies, Inc.; Invitrogen), and cells were harvested 48 h after transfection. Three independent experiments at different days for each assay were conducted.

Blood glucose, serum insulin, GTT, ITT, and homeostasis model assessment of insulin resistance index. Blood glucose levels were measured using a Glucometer Elite monitor. Serum insulin levels were measured using the Mercodia Ultrasensitive Rat Insulin ELISA kit (ALPCO Diagnostic). GTTs and ITTs were performed by intraperitoneal injection of 2 g/kg glucose after overnight fasting and 0.75 units/kg insulin after 4 h fasting, respectively. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated according to the following formula: [fasting glucose levels (mmol/L)] × [fasting serum insulin (μU/mL)]/22.5. Area under curve was calculated as previously described (21).

In vivo insulin signaling assay. Mice were fasted for 6 h before insulin injection, and insulin signaling in livers was assayed as previously described (22).

RNA isolation and relative quantitative RT-PCR. RT-PCR was performed as previously described (20). The sequences of primers used in this study are available upon request.

Western blot analysis. Western blot analysis was performed as previously described (22). Primary antibodies (anti-ATF4 and anti-TRB3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti–p-IR, anti–total-IR, anti–p-AKT, anti–total-AKT, anti–p-mTOR, anti–total-mTOR, anti–p-p70S6K1, anti–total-p70S6K1, anti–p-S6, anti–total-S6, anti–p-eIF2α, anti–total-eIF2α, anti–p-PERK and anti–total-PERK (all the above from Cell Signaling Technology, Beverly, MA), and antiactin antibody (Sigma-Aldrich) were incubated overnight at 4°C, and specific proteins were visualized by ECL Plus (Amersham Biosciences, Buckinghamshire, U.K.). Band intensities were measured using Quantity One (Bio-Rad Laboratories, Hercules, CA) and normalized to total protein or actin.

Immunohistochemistry staining. Immunohistochemistry staining was performed as described previously (23). In brief, brain coronal sections of 25 μm were cut using a frozen microtome (Leica Microsystems, Wetzlar, Germany) and incubated with anti-ATF4 antibody, and pictures were taken using an Olympus BX61 microscope (Olympus, Tokyo, Japan).

Statistical analysis. All data are expressed as means ± SEM, which are representative of at least three independent in vitro experiments or at least two independent in vivo experiments, with the numbers of mice included in each group in each experiment indicated. Significant differences were assessed by two-tailed Student t test or one-way ANOVA followed by the Student-Newman-Keuls (SNK) test. $P < 0.05$ was considered statistically significant.

RESULTS

Activation of hypothalamic ATF4 by intracerebroventricular injection of Ad-ATF4 induces hepatic insulin resistance.
To investigate the possible involvement of hypothalamic ATF4 in the regulation of insulin sensitivity,

we intracerebroventricularly injected mice with Ad-ATF4 or adenovirus expressing green fluorescent proteins (Ad-GFP) as a control. Overexpression of ATF4 in the hypothalamus was confirmed by Western blotting and RT-PCR analysis, and the effects of Ad-ATF4 were demonstrated by the increased expression of tribbles homolog 3 (TRB3), one of the well-known targets of ATF4 (24), in the hypothalamus, compared with the Ad-GFP group (Fig. 1A and B). Immunohistochemistry staining showed that levels of ATF4 were mainly increased in the arcuate nucleus (Arc), along the ventricle in the hypothalamus, and less abundantly in the paraventricular nucleus of the hypothalamus (PVN), compared with Ad-GFP mice (Fig. 1C).

The effects of intracerebroventricular injection of Ad-ATF4 were then examined. Although intracerebroventricular injection of Ad-ATF4 had no effect on fed blood glucose and serum insulin levels, fasting blood glucose and serum insulin levels were increased significantly in Ad-ATF4 mice compared with the Ad-GFP group (Fig. 2A and B). Consistently, the HOMA-IR index was increased in these mice (Fig. 2C). Intracerebroventricular injection of Ad-ATF4 significantly induced glucose intolerance and attenuated glucose clearance, as demonstrated by GTTs and ITTs, respectively,
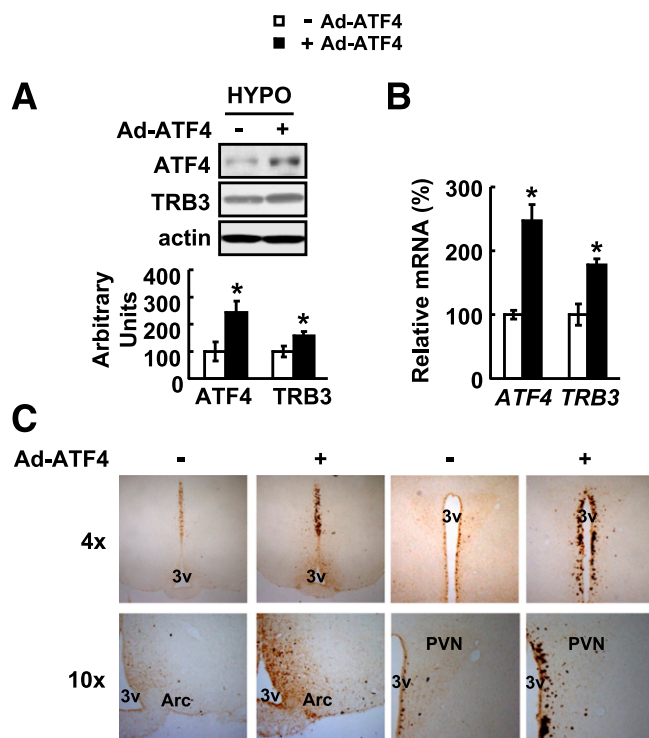

FIG. 1. Intracerebroventricular injection of Ad-ATF4 increases ATF4 expression in the hypothalamus. Mice received intracerebroventricular injection of Ad-ATF4 (+Ad-ATF4) or green fluorescent protein (−Ad-ATF4) prior to examination of ATF4 and downstream target TRB3 expression on day 7 post adenoviral injection. Means ± SEM shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (Ad-GFP group, n = 7; Ad-ATF4 group, n = 7 for A and B; Ad-GFP group, n = 6; Ad-ATF4 group, n = 6 for C). Statistical significance was calculated by two-tailed Student t test: *P < 0.05 (for the effect of with vs. without Ad-ATF4). A: Hypothalamic ATF4 and TRB3 protein (top, Western blot; bottom, quantitative measurements of ATF4 and TRB3 protein relative to actin). B: Hypothalamic Atf4 and Trb3 mRNA. C: Immunohistochemistry staining for ATF4 in hypothalamus. 3v, third ventricle. Images shown are representative of several animals for each group. (A high-quality color representation of this figure is available in the online issue.)

compared with the Ad-GFP group (Fig. 2D and E and Supplementary Fig. 1A).

Decreased insulin sensitivity in mice suggests a decrease in insulin sensitivity in peripheral tissues, including liver. The neural connection between the hypothalamus and the liver plays an important role in the CNS control of systematic glucose homeostasis (4,6). Based on this knowledge, we examined the phosphorylation levels of two key components in the insulin signaling pathways, insulin receptor (IR) and protein kinase B (AKT), in the liver after infusion of insulin (2 units/kg) into the hepatic portal vein, as described previously (22). As expected, overexpression of hypothalamic ATF4 significantly impaired insulin-stimulated phosphorylation of IR and AKT in the liver compared with the Ad-GFP group (Fig. 2F).

Inhibition of ATF4 in the hypothalamus by intracerebroventricular injection of adenovirus expressing a dominant-negative ATF4 variant (Ad-DN-ATF4) improves hepatic insulin sensitivity. We then examined the effect of hypothalamic ATF4 inhibition via intracerebroventricular injection of Ad-DN-ATF4, which is recognized as an ATF4-specific inhibitor (18), or Ad-GFP as a control. Virus-mediated gene expression of Ad-DN-ATF4 was verified by increased ATF4 expression and decreased expression of TRB3, compared with the Ad-GFP group (Fig. 3A and B). Intracerebroventricular injection of Ad-DN-ATF4 had no effect on levels of blood glucose and

serum insulin in the fed state; however, it greatly decreased fasting serum insulin levels, although fasting blood glucose levels were unaffected, compared with the Ad-GFP group (Fig. 3C and D). Consistently, the HOMA-IR index was also decreased significantly in these mice (Fig. 3E). Glucose tolerance and insulin sensitivity were greatly improved by intracerebroventricular injection of Ad-DN-ATF4 compared with the control group, as evaluated by GTTs and ITTs, respectively (Fig. 3F and G and Supplementary Fig. 1B). Consistent with these changes, intracerebroventricular injection of Ad-DN-ATF4 also significantly enhanced insulin-stimulated phosphorylation of IR and AKT in the liver compared with the Ad-GFP group (Fig. 3H).

Selective hepatic vagotomy reverses hypothalamic ATF4-induced insulin resistance in the liver. Previous studies have shown that vagus nerve innervations are critical in mediating the CNS regulation of hepatic glucose homeostasis (4,6,7), suggesting a possible involvement of the vagus nerve in hypothalamic ATF4-induced hepatic insulin resistance. To test this hypothesis, we performed selective hepatic branch vagotomy or sham surgery (isolation of the nerve without resection) in mice (4), prior to intracerebroventricular injection of Ad-ATF4 or Ad-GFP.

As expected, intracerebroventricular injection of Ad-ATF4 increased ATF4 and TRB3 protein levels in the hypothalamus
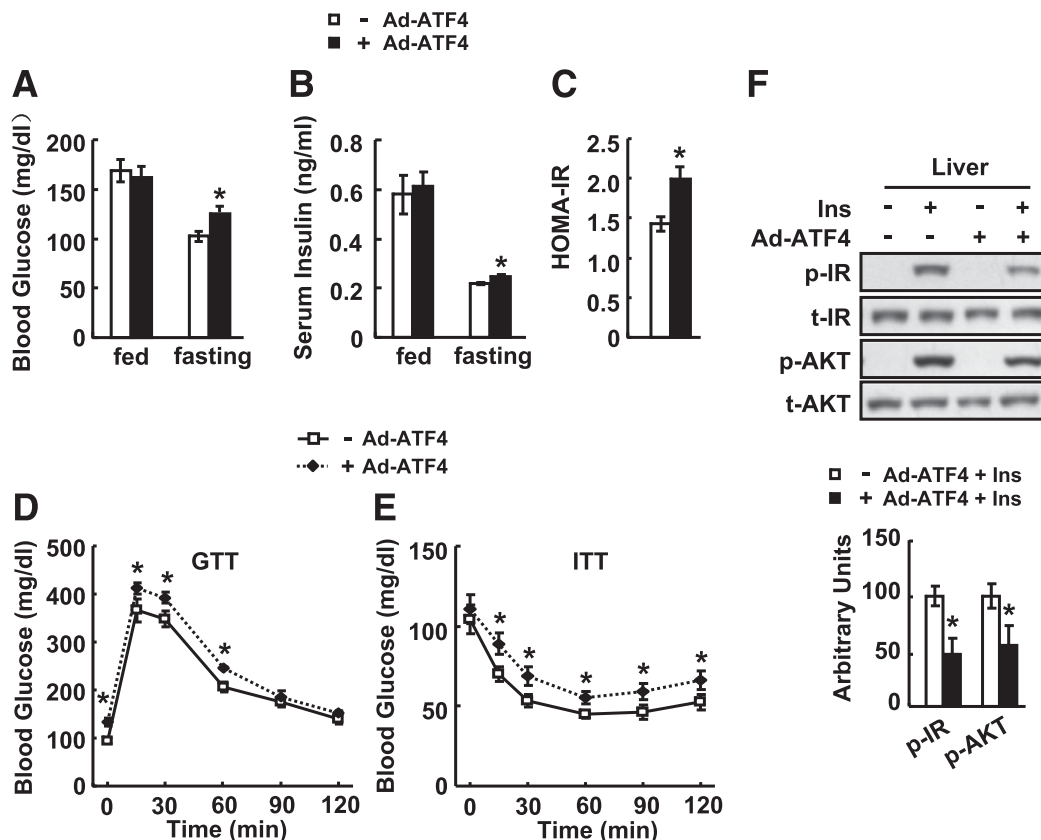

FIG. 2. Activation of hypothalamic ATF4 by intracerebroventricular injection of Ad-ATF4 induces hepatic insulin resistance. Mice received intracerebroventricular injection of adenovirus expressing Ad-ATF4 (+Ad-ATF4) or green fluorescent protein (−Ad-ATF4) prior to all measurements on day 7 post adenoviral injection. Hepatic insulin signaling was examined before (−Ins) and after (+Ins) 2 units/kg insulin stimulation for 3 min. Means ± SEM shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (n = 6 or 7 for each group). Statistical significance was calculated by two-tailed Student t test: *P < 0.05 (for the effect of with vs. without Ad-ATF4). A: Blood glucose levels. B: Serum insulin levels. C: HOMA-IR index. D: GTT. E: ITT. F: p-IR and p-AKT protein in liver (top, Western blot; bottom, quantitative measurements of p-IR and p-AKT protein relative to their total protein).
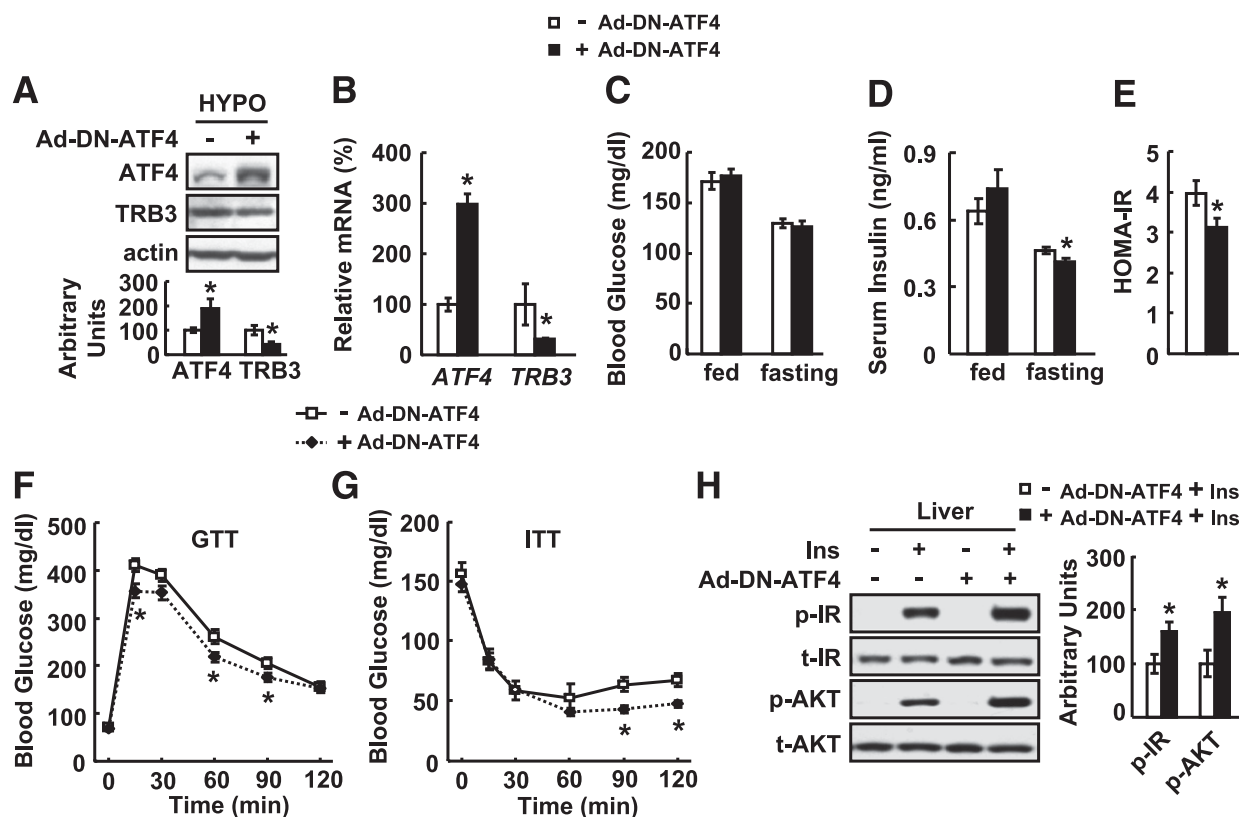
FIG. 3. Inhibition of hypothalamic ATF4 by intracerebroventricular injection of Ad-DN-ATF4 improves hepatic insulin sensitivity. Mice received intracerebroventricular injection of adenovirus expressing Ad-DN-ATF4 (+Ad-DN-ATF4) or green fluorescent protein (−Ad-DN-ATF4) prior to all measurements on day 7 post adenoviral injection. Hepatic insulin signaling was examined before (−Ins) and after (+Ins) 2 units/kg insulin stimulation for 3 min. Means ± SEM shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated ($n = 6$ for each group). Statistical significance was calculated by two-tailed Student t test: *$P < 0.05$ (for the effect of with vs. without Ad-DN-ATF4). A: Hypothalamic ATF4 and TRB3 protein (*top*, Western blot; *bottom*, quantitative measurements of ATF4 and TRB3 protein relative to actin). B: Hypothalamic *Atf4* and *Trb3* mRNA. C: Blood glucose levels. D: Serum insulin levels. E: HOMA-IR index. F: GTT. G: ITT. H: p-IR and p-AKT protein in liver (*left*, Western blot; *right*, quantitative measurements of p-IR and p-AKT protein relative to their total protein).

compared with the Ad-GFP group (Fig. 4A). Consistent with previous results (Fig. 1), intracerebroventricular injection of Ad-ATF4 had no effect on blood glucose and serum insulin levels in the fed state, which were also not affected by hepatic vagotomy compared with control mice (Fig. 4B and C). By contrast, increased fasting blood glucose levels by Ad-ATF4 were largely decreased by hepatic vagotomy compared with sham surgery, although increased fasting insulin levels by Ad-ATF4 were not changed by hepatic vagotomy (Fig. 4B and C). The increased HOMA-IR index by Ad-ATF4 was also significantly reduced by hepatic vagotomy (Fig. 4D). Consistent with these changes, Ad-ATF4–impaired glucose tolerance and clearance, as well as insulin signaling in the liver, were also largely reversed by hepatic vagotomy (Fig. 4E–G and Supplementary Fig. 1C).

ATF4 stimulates hypothalamic mTOR/S6K1 phosphorylation in vitro and in vivo. Previous studies have shown that activation of the mTOR downstream target S6K1 in the hypothalamus contributes to hepatic insulin resistance (5) and that mTOR/S6K1 activity is decreased in the livers and white adipose tissue (WAT) of ATF4 KO mice (11). These results raised the possibility that hypothalamic ATF4 may act through mTOR/S6K1 signaling to mediate hepatic insulin resistance.

To test this hypothesis, we examined the effects of knocking down ATF4 expression on the phosphorylation levels of mTOR and S6K1 in primary cultured hypothalamic neurons transfected with plasmid encoding a dominant-negative mutant of ATF4 (DN-ATF4) or control vector. Consistent with previous reports (11), inhibition of ATF4 significantly decreased phosphorylation of mTOR and S6K1 in primary cultured hypothalamic neurons compared with control cells (Fig. 5A). We then examined the effects of ATF4 overexpression by transfecting primary cultured hypothalamic neurons with plasmid encoding ATF4 or control vector. As expected, overexpression of ATF4 stimulated phosphorylation of mTOR and S6K1 (Fig. 5B). Based on these observations, we examined this relationship between ATF4 and S6K1 phosphorylation in vivo and similar results were obtained (Fig. 5C and D).

Intracerebroventricular injection of Ad-shS6K1 reverses hypothalamic ATF4-induced hepatic insulin resistance. A role for mTOR/S6K1 signaling in mediating hypothalamic ATF4-induced hepatic insulin resistance was investigated in mice intracerebroventricularly injected with Ad-ATF4 or Ad-GFP, simultaneously injected with Ad-shS6K1 or control scrambled adenovirus (Ad-scramble). Functional validation of Ad-shS6K1 was demonstrated by its blocking effect on Ad-ATF4–induced increases in phosphorylation of the S6K1 downstream target S6 in the hypothalamus in the presence or absence of intracerebroventricular injection of Ad-ATF4 (Fig. 6A). Although no differences in fed blood glucose and serum insulin levels were observed among different groups,
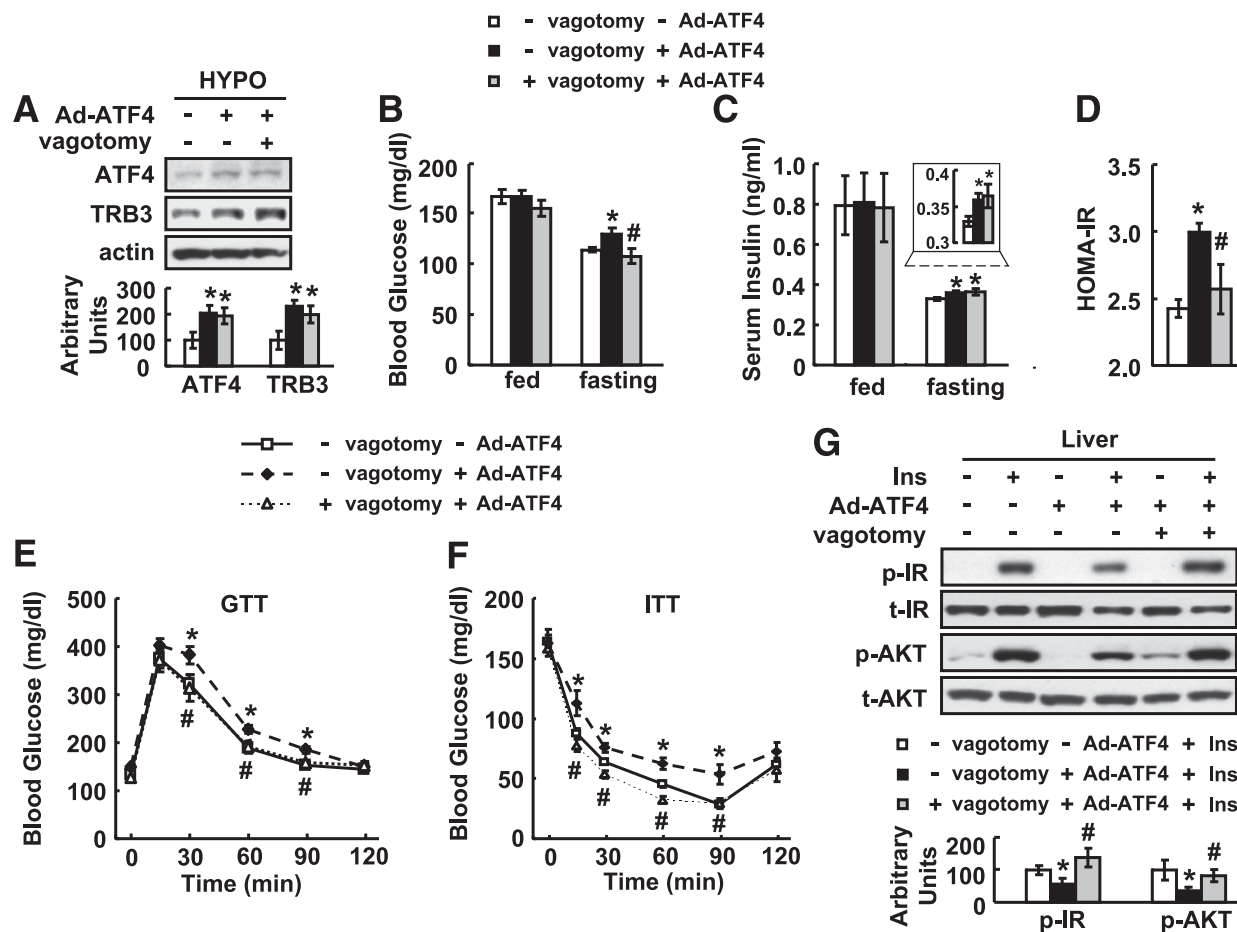
FIG. 4. Selective hepatic vagotomy reverses hypothalamic ATF4-induced hepatic insulin resistance. Mice were subjected with selective hepatic branch vagotomy (+vagotomy) or sham surgery (−vagotomy), followed by intracerebroventricular injection with adenovirus expressing Ad-ATF4 (+Ad-ATF4) or green fluorescent protein (−Ad-ATF4), prior to all measurements on day 7 post adenoviral injection. Hepatic insulin signaling was examined before (−Ins) and after (+Ins) 2 units/kg insulin stimulation for 3 min. Means ± SEM shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (sham with Ad-GFP group, $n = 6$; sham with Ad-ATF4 group, $n = 7$; vagotomy with Ad-ATF4 group, $n = 7$). Statistical significance was calculated by one-way ANOVA followed by the SNK test: *$P < 0.05$ (for the effect of any group vs. without Ad-ATF4) and #$P < 0.05$ (for the effect of with vs. without vagotomy in the Ad-ATF4 group). *A*: Hypothalamic ATF4 and TRB3 protein (*top*, Western blot; *bottom*, quantitative measurements of ATF4 and TRB3 protein relative to actin). *B*: Blood glucose levels. *C*: Serum insulin levels. *D*: HOMA-IR index. *E*: GTT. *F*: ITT. *G*: p-IR and p-AKT protein in liver (*top*, Western blot; *bottom*, quantitative measurements of p-IR and p-AKT protein relative to their total protein).

fasting blood glucose and serum insulin levels were increased significantly in Ad-ATF4 mice compared with the Ad-GFP group, and Ad-shS6K1 prevented this upregulation (Fig. 6*B* and *C*). Consistent with these changes, the Ad-ATF4–mediated increased HOMA-IR index was also decreased by inhibition of S6K1 activity (Fig. 6*D*). As mentioned above (Fig. 1), intracerebroventricular injection of Ad-ATF4, in the presence of Ad-scramble, also impaired glucose tolerance and clearance compared with control mice coinjected with Ad-scramble and Ad-GFP (Fig. 6*E* and *F*). Consistent with a role for S6K1 in mediating the effect of ATF4 on insulin sensitivity, intracerebroventricular injection of Ad-shS6K1 largely prevented Ad-ATF4–induced impairment in glucose tolerance and clearance and insulin signaling in the liver (Fig. 6*E–G* and Supplementary Fig. 1*D*). Ad-shS6K1 alone decreased fasting serum levels compared with control mice; the HOMA-IR index was not significantly changed, which could be due to unchanged fasting blood glucose levels in these mice (Fig. 6*B–D*). Except for this, Ad-shS6K1 alone treatment also improved insulin sensitivity (Fig. 6*A–G*).

Inhibition of ATF4 in the hypothalamus by intracerebroventricular injection of Ad-DN-ATF4 reverses acute brain ER stress–induced hepatic insulin resistance. Based on the above results, we speculated that ATF4 might also be involved in mediating acute brain ER stress–induced hepatic insulin resistance as shown recently (3), as ATF4 has been shown to be involved in the ER stress response in different models (16). Because of the complexity of ER stress cascades (16), we used pharmacological strategies to induce brain ER stress by intracerebroventricular injection of TG, a classical ER stress–inducing chemical that has been extensively used (3), in mice intracerebroventricularly injected with Ad-GFP or Ad-DN-ATF4.

Consistent with previous reports (3), intracerebroventricular injection of TG increased phosphorylation of PERK and eIf2α, well known as ER stress markers (16), as well as expression levels of ATF4 (Fig. 7*A*). The possible involvement of ATF4 in mediating brain ER stress–induced hepatic insulin resistance was then investigated in mice intracerebroventricularly injected with Ad-DN-ATF4 or Ad-GFP, followed by intracerebroventricular injection of TG. TG treatment had no effect on fed blood glucose and serum
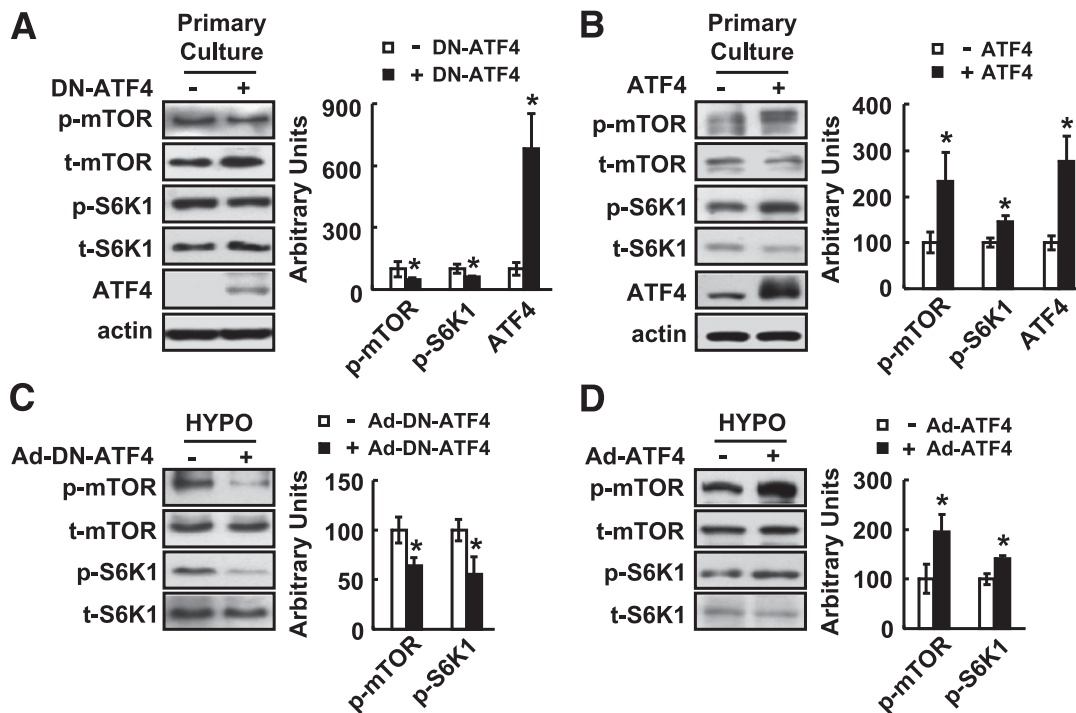
FIG. 5. Hypothalamic ATF4 regulates phosphorylation of mTOR/S6K1 in vitro and in vivo. *A* and *B*: Primary cultured hypothalamic neurons were transfected with plasmid encoding a dominant-negative variant of ATF4 (+DN-ATF4) or control vector (−DN-ATF4), or plasmid encoding ATF4 (+ATF4) or control vector (−ATF4). *C* and *D*: Mice received intracerebroventricular injection of adenovirus expressing Ad-DN-ATF4 (+Ad-DN-ATF4) or green fluorescent protein (−Ad-DN-ATF4), or adenovirus expressing Ad-ATF4 (+Ad-ATF4) or green fluorescent protein (−Ad-ATF4). Means ± SEM shown are representative of at least three independent in vitro experiments or two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (*n* = 6 for each group). Statistical significance was calculated by two-tailed Student *t* test: *$P < 0.05$ (for the effect of overexpression or inhibition of ATF4 vs. control group). *A* and *B*: p-mTOR, p-S6K1, and ATF4 protein in primary cultured hypothalamic neurons (*left*, Western blot; *right*, quantitative measurements of p-mTOR, p-S6K1, and ATF4 protein relative to their total protein or actin). *C* and *D*: Hypothalamic p-mTOR and p-S6K1 protein (*left*, Western blot; *right*, quantitative measurements of p-mTOR and p-S6K1 protein relative to their total protein).

insulin levels in any group (Fig. 7*B* and *C*). By contrast, it significantly increased fasting blood glucose and serum insulin levels, which were largely reversed by intracerebroventricular injection of Ad-DN-ATF4 (Fig. 7*B* and *C*). The increased HOMA-IR index by TG treatment was also decreased in these mice (Fig. 7*D*). GTT and ITT tests were performed 3 days after TG treatment. As shown previously (3), TG treatment significantly impaired glucose tolerance and clearance and insulin signaling in the liver, and these effects of TG treatment were largely reversed by intracerebroventricular injection of Ad-DN-ATF4 (Fig. 7*E*–*G* and Supplementary Fig. 1*E*). Similar to the above observations (Fig. 5), TG treatment–increased S6 phosphorylation was largely blocked by Ad-DN-ATF4 compared with the control group (Fig. 7*H*).

DISCUSSION

Previous studies have suggested the involvement of ATF4 in a variety of metabolic responses (11–14); however, a role of hypothalamic ATF4 in the regulation of hepatic insulin sensitivity has not previously been reported. In this study, we used an intracerebroventricular injection technique to overexpress or inhibit ATF4 expression in the hypothalamus in mice. Our results show that overexpression of hypothalamic ATF4 blunts hepatic insulin signaling in mice, whereas inhibition of ATF4 has the opposite effect. Furthermore, we found that the signal from hypothalamic ATF4 is mediated via the hepatic branch of the vagus nerve. Taken together, our findings describe

a novel ATF4-mediated pathway for CNS regulation of insulin sensitivity in the liver and show that contribution of CNS should not be ignored when investigating insulin resistance in the peripheral tissues.

The hypothalamus regulates target tissues via the autonomic nervous system, with the hepatic branch of the vagus nerve providing the primary communication link with the liver (4,6,7). Accumulating evidence suggests that the autonomic nervous system plays an essential role in CNS regulation of hepatic glucose metabolism and insulin sensitivity (4,6,7,25). For example, the suppression of glucose production by central administration of insulin or fatty acids is largely abolished by selective hepatic vagal denervation (4,7). We speculate that ATF4 may regulate insulin sensitivity via a neural route from the hypothalamus to the liver, and we are unique in demonstrating this hypothesis. The requirement of the vagus nerve in response to central ATF4 signaling is also consistent with its critical role in mediating effects of intracerebroventricular dexamethasone or hypothalamic leptin in the regulation of peripheral insulin sensitivity (26,27). Furthermore, the altered vagus activity by hypothalamic ATF4 might be mediated by ATF4 modulation of insulin signaling in the brain, which has previously been linked to the regulation of vagus activity (4,7,28), as both of our in vitro and in vivo experiments have shown that ATF4 can regulate insulin signaling in the brain (data not shown).

The signaling by mTOR and S6K1 has been shown to be essential for protein synthesis, growth, and development (29). Activation of mTOR/S6K1 signaling produces insulin
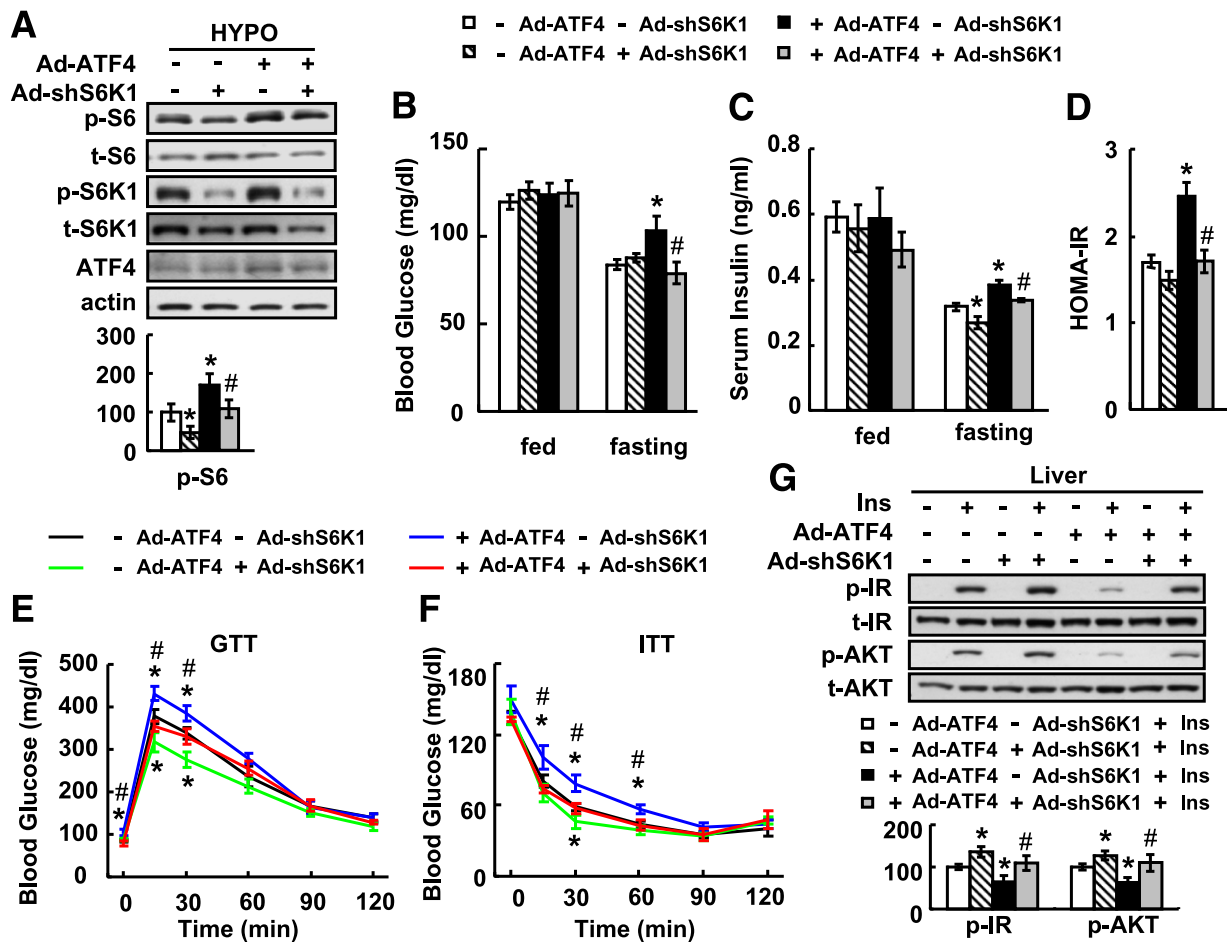
FIG. 6. Inhibition of hypothalamic S6K1 by intracerebroventricular injection of Ad-shS6K1 reverses hypothalamic ATF4-induced hepatic insulin resistance. Mice received intracerebroventricular injection of Ad-ATF4 (+Ad-ATF4) or Ad-GFP (−Ad-ATF4) and Ad-shS6K1 (+Ad-shS6K1) or scrambled adenovirus (−Ad-shS6K1) prior to measurements on day 7 post adenoviral injection. Hepatic insulin signaling was examined before (−Ins) and after (+Ins) 2 units/kg insulin stimulation for 3 min. Means ± SEM shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (−Ad-shS6K1 −Ad-ATF4, n = 8; +Ad-shS6K1 −Ad-ATF4, n = 7; −Ad-shS6K1 +Ad-ATF4, n = 8; +Ad-shS6K1 +Ad-ATF4, n = 5). Statistical significance was calculated by one-way ANOVA followed by the SNK test: *P < 0.05 (for the effect of any group vs. without Ad-ATF4) and #P < 0.05 (for the effect of with vs. without Ad-shS6K1 in Ad-ATF4 group). A: Hypothalamic p-S6, p-S6K1, and ATF4 protein (*left*, Western blot; *right*, quantitative measurements of p-S6 protein relative to its total protein). B: Blood glucose levels. C: Serum insulin levels. D: HOMA-IR index. E: GTT. F: ITT. G: p-IR and p-AKT protein in liver (*top*, Western blot; *bottom*, quantitative measurements of p-IR and p-AKT protein relative to their total protein).

resistance in various cell lines (30,31) via directly phosphorylating several serine residues on insulin receptor substrate 1 (IRS1) (30). Increased IRS1 serine phosphorylation reduces the activity of IRS1, thereby impairing PI3K/AKT signaling and increasing insulin resistance (32,33). Consistent with a role for S6K1 in insulin sensitivity, S6K1 activity is increased in the livers of *db/db* mice and in HFD-induced insulin-resistant animal models (34), whereas deletion of S6K1 in mice enhances insulin sensitivity (35,36). The importance of hypothalamic S6K1 in the regulation of hepatic insulin resistance is revealed by a recent study showing that activation of S6K1 in the hypothalamus induces hepatic insulin resistance, and inhibition of S6K1 activity in the hypothalamus ameliorates HFD-induced hepatic insulin resistance (5). These results raised the possibility that hypothalamic S6K1 may mediate central ATF4-induced insulin resistance in the liver.

This possibility was first confirmed by our observation on the regulatory effects of ATF4 on S6K1 activity in vitro and in vivo, results consistent with a previous study

showing that S6K1 activity is decreased in the livers and WAT of ATF4-deficient mice (11). A key role for S6K1 in mediating ATF4 effects on insulin sensitivity is then confirmed in our current study by the reversal effect of Ad-shS6K1 on hypothalamic ATF4-induced insulin resistance in the liver. Ad-shS6K1 alone also has similar effects in improving hepatic insulin sensitivity. These results are consistent with previous observations that S6K1-deficient mice are more sensitive to insulin than wild-type mice (36) and inhibition of S6K1 in the hypothalamus improves insulin sensitivity (5). However, the reported effects of hypothalamic S6K1 on insulin sensitivity are not always the same. In contrast to our results and those of Ono et al. (5), another study reported that activation of hypothalamic S6K1 reverses insulin resistance by HFD (37). We believe, however, that this phenotype is most likely caused by the significantly decreased food intake and body weight, rather than a direct effect of hypothalamic S6K1 on insulin sensitivity (37). Thus, our study not only demonstrates a role for S6K1 in mediating the hypothalamic ATF4 effect on hepatic insulin sensitivity but also supports a role for
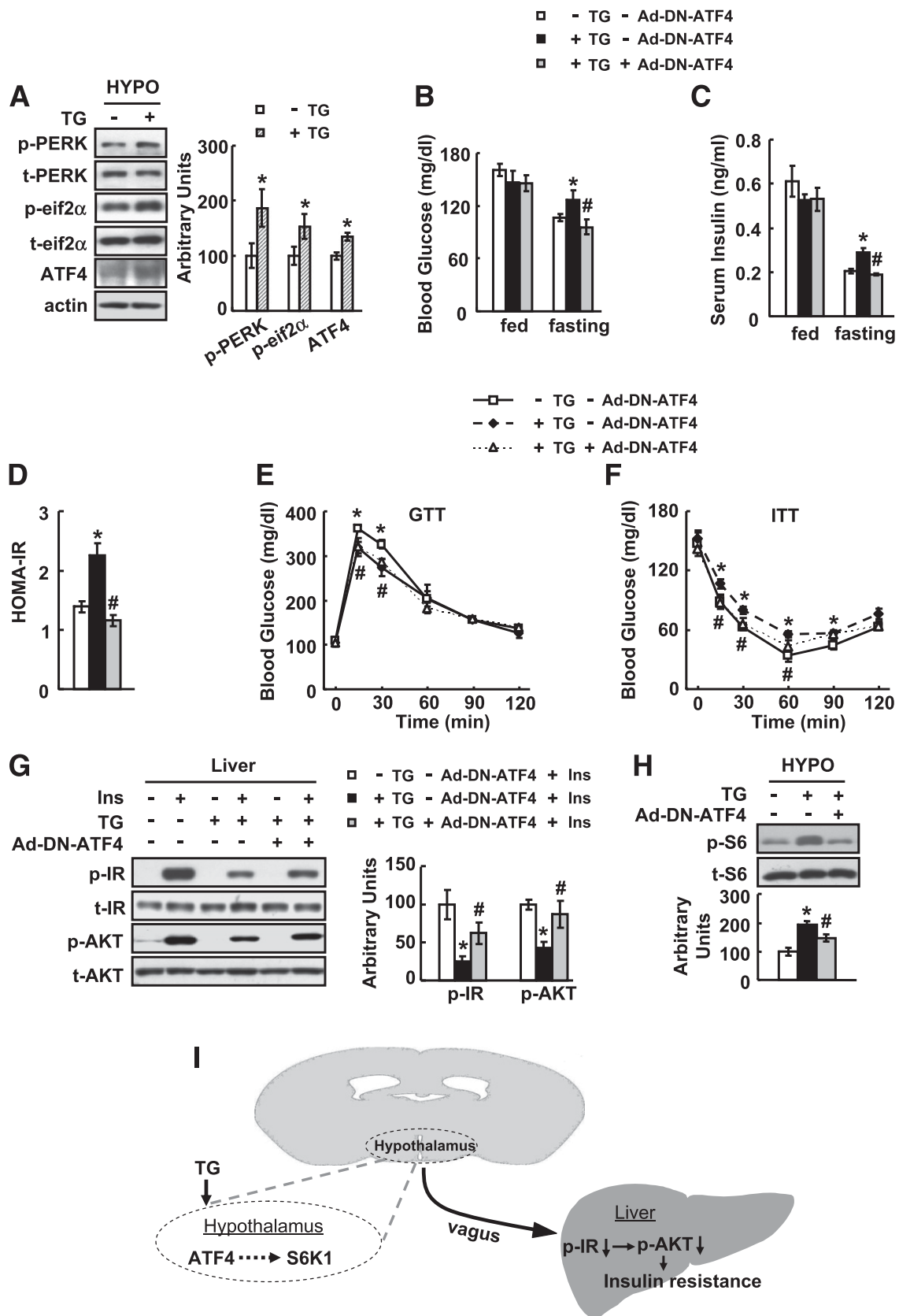
FIG. 7. Inhibition of hypothalamic ATF4 reverses hepatic insulin resistance induced by acute brain ER stress. Mice received intra-cerebroventricular injection of Ad-DN-ATF4 (+Ad-DN-ATF4) or Ad-GFP (−Ad-DN-ATF4), followed by treatment with 1 μg/day TG (+TG) or without (−TG) for three consecutive days, prior to all measurements on day 7 post adenoviral injection. Hepatic insulin signaling was examined before (−Ins) and after (+Ins) 2 units/kg insulin stimulation for 3 min. Means ± SEM shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiment indicated (DMSO with Ad-GFP group, $n = 5$; TG with Ad-GFP group, $n = 6$; TG with Ad-DN-ATF4 group, $n = 6$). Statistical significance was calculated by two-tailed Student t test: *$P < 0.05$ (for the effect of with

hypothalamic S6K1 in regulating peripheral insulin sensitivity.

A variety of intracellular stresses have been recognized as primary pathogenic factors for insulin resistance (38), including ER stress, which initiates a set of intracellular responses that interfere with the normal function of the ER (39–41). Increased ER stress has been observed in insulin-secreting pancreatic β-cells (39) and additional insulin-responsive peripheral tissues (40,41). Recent studies have shown that ER stress is also induced in the hypothalamus by overnutrition (15) and contributes to insulin resistance directly via altered NF-κB signaling in the hypothalamus (3) or indirectly by promoting feeding and weight gain (15,42).

ATF4 is a well-known downstream effector for ER stress (16). In the current study, we used pharmacological approaches to induce acute brain ER stress to show for the first time that hypothalamic ATF4 mediates brain ER stress–induced hepatic insulin resistance, most likely via an mTOR/S6K1 pathway. As increased brain ER stress is also observed in mice under HFD (15), this finding suggests that ATF4 might be involved in the regulation of this chronic ER stress–induced insulin resistance and obesity. Moreover, pathways directed by inositol-requiring enzyme 1/x-box–binding protein 1 and ATF6 are also involved in the ER stress response (16) and insulin sensitivity regulation (39,40). Thus, our results suggest that these signaling pathways might also have the potential to mediate brain ER stress–regulated peripheral metabolic changes. In the current study, we did not observe TG-induced activation of caspase-12 (data not shown), a crucial signal of apoptosis induced by ER stress (43). We do not exclude the possibility of apoptosis, however, if the duration of TG treatment was prolonged as shown previously in another study (44).

In the current study, the mechanisms by which ATF4 regulates mTOR/S6K1 signaling are unclear. Studies have shown that mTOR/S6K1 activity could be modulated by AMP-activated protein kinase (AMPK), which functions as an intracellular nutrient sensor to control protein synthesis, cell growth, and metabolism (45). We therefore speculated that AMPK might be involved in the ATF4 regulation of S6K1 activity in the hypothalamus. Consistent with these results, we found that AMPK phosphorylation was inhibited by hypothalamic ATF4, and ATF4-stimluated phosphorylation of mTOR and S6K1 was significantly blocked by constitutively active AMPKα1 (46) in vitro (Supplementary Fig. 2). Because AMPK activity has been shown to be influenced by SCD1 (47), and SCD1 expression is regulated by ATF4 in other tissues (12,14) and the hypothalamus (data not shown), we speculate that ATF4 may regulate AMPK phosphorylation via modulation of SCD1 expression in vivo. This possibility will be investigated in the future.

In addition to the direct link between the brain and liver, changes in food intake and body weight and the other metabolic tissues, muscle and WAT, can also contribute to insulin sensitivity in the whole body and liver. However, these factors are unlikely to make a major contribution to the effects of hypothalamic ATF4 on hepatic insulin sensitivity, since food intake, body weight, and fat mass were only increased ~8, 4, and 8%, respectively, by Ad-ATF4 (Supplementary Fig. 3), and insulin-stimulated phosphorylation of IR and AKT was also not detected earlier in muscle and WAT than in liver in Ad-ATF4 mice (unpublished data).

Another important issue that remains to be answered concerns the molecular targets of ATF4 signaling in the CNS. Candidates include Arc and PVN, which have recently been shown to regulate glucose metabolism and insulin sensitivity via the autonomic nervous system connections with the liver (25,48,49). This possibility is supported by our observations that exogenous ATF4 expression is observed primarily in the Arc, and less abundantly in the PVN. Additional regions adjacent to the ventricle also showed ATF4 staining, however, suggesting that nuclei in these areas might also be involved in central ATF4 signaling. The specific neurons involved are currently under investigation.

In summary, the experiments described in this article show that hypothalamic ATF4 regulates hepatic insulin sensitivity via the vagus nerve, and the downstream effects of ATF4 are mediated via mTOR/S6K1 signaling. Our experiments further show that inhibition of ATF4 in the hypothalamus reverses acute brain ER stress–induced hepatic insulin resistance (Fig. 7I). Taken together, these results demonstrate a novel function for hypothalamic ATF4 in the regulation of insulin sensitivity in peripheral tissues, thereby providing a new perspective for our understanding of CNS regulation of peripheral insulin sensitivity. The important role of ATF4 in brain ER stress–induced hepatic insulin resistance also largely expands our understanding of the broad role that ATF4 plays in the regulation of metabolism.

vs. without TG treatment) in *A*, or by one-way ANOVA followed by the SNK test: *$P < 0.05$ (for the effect of any group with vs. without TG) and #$P < 0.05$ (for the effect of with vs. without Ad-DN-ATF4 under TG treatment) in *B–H*. *A*: Hypothalamic p-PERK, p-eIF2α, and ATF4 protein (*left*, Western blot; *right*, quantitative measurements of p-PERK, p-eIF2α, and ATF4 protein relative to their total protein or actin). *B*: Blood glucose levels. *C*: Serum insulin levels. *D*: HOMA-IR index. *E*: GTT. *F*: ITT. *G*: p-IR and p-AKT protein in liver (*left*, Western blot; *right*, quantitative measurements of p-IR and p-AKT protein relative to their total protein). *H*: Hypothalamic p-S6 protein (*top*, Western blot; *bottom*, quantitative measurements of p-S6 relative to its total protein). *I*: Working model.

the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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