Central activating transcription factor (ATF4) regulates hepatic insulin resistance in mice via S6K1 signaling and the vagus nerve

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Running Title: CNS ATF4 controls hepatic insulin sensitivity

The word count: 4217

The number of tables: 0

The number of figures: 7
Abstract

Recent studies have revealed that the central nervous system (CNS), 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 (ATF) 4 in the regulation of insulin sensitivity in the liver. Here, we show that overexpression of ATF4 in the hypothalamus resulting from intraventricular (icv) injection of adenovirus expressing ATF4 induces hepatic insulin resistance in mice and that inhibition of hypothalamic ATF4 by icv 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 demonstrate 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.

Introduction

The incidence of type 2 diabetes (T2D) 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-kB have been shown to contribute to insulin resistance in the liver (3; 5). It has also been shown that 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 (ATF) 4 belongs to the cAMP-response element-binding protein (CREB) 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 (HCD)-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 present 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/6 J 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 CO₂ inhalation. Tissues were isolated and snap-frozen and stored at -80°C for future analysis. Normally, the same set of mice were used for measuring levels of fasting blood glucose and serum insulin, 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 were used for measuring fed blood glucose levels prior to fasting, 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, Sibs, CAS.

**Generation of recombinant adenoviruses.**

The ATF4 plasmid (17) and the plasmid encoding dominant negative mutant of ATF4 (DN-ATF4) (18) were kindly provided by Dr. Zaiqing Yang (Huazhong Agricultural University, Wuhan, P. R. China) and Dr. Jawed Alam (Louisiana State University Health Sciences Center, LA, USA), 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 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-iTTM Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. The sequence designed for the knockdown of S6K1 is: 5’-CACCGGGAGTTGGACCATATGAACTCGAAAGTTCATATGGTCCAACTCC-3’.
**Intracerebroventricular (icv) administration experiments.**

Icv administration experiments were conducted as previously described (3). 1 µl 4x10^8 pfu/mice of Ad-ATF4, Ad-DN-ATF4, Ad-shS6K1 or control adenovirus were 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 micro syringe. A 7 day post-adenoviral 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, that was back towards baseline at 14 and 21 days. For thapsigargin (TG) administration experiments, mice were icv injected with Ad-DN-ATF4 or Ad-GFP and implanted with cannula, then allowed to recover for 3 days. Following recovery, mice were injected with 2 µl of 0.5 µg/µl TG (Sigma, MO, USA, dissolved in artificial cerebrospinal fluid (aCSF) containing 10% DMSO) or control vehicle once daily for 3 consecutive days, as previously described (3).

**Selective Hepatic Vagotomy.**

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

**Primary hypothalamic neurons 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, Carlsbad, CA) 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 HOMA-IR 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 IP injection of 2 g/kg glucose after overnight fasting and 0.75 u/kg insulin after 4 h fasting, respectively. HOMA-IR index was calculated according to the formula: [fasting glucose levels (mmol/L)] x [fasting serum insulin (μ U/ml)]/ 22.5. Area under curve (AUC)s were calculated as previously described (21).

**In vivo insulin signaling assay.**

Mice were fasted for 6 h before insulin injection and insulin signaling in livers were 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 (from Santa Cruz Biotechnology, Inc., CA, USA), 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, MA, USA), and anti-actin antibody (from Sigma, MO, USA) were incubated overnight at 4 °C and specific proteins were visualized by ECL Plus (Amersham Biosciences, Buckinghamshire, UK). Band intensities were measured using Quantity One (Bio-Rad Laboratories, CA, USA) and normalized to total protein or actin.

Immunohistochemistry (IHC) staining.

IHC staining was performed as described previously (23). Briefly, brain coronal sections of 25 μm were cut using a frozen microtome (Leica Microsystems, Germany), incubated with anti-ATF4 antibody, and pictures were taken by using an Olympus BX61 microscope (Olympus, 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 test. P < 0.05 was considered statistically significant.

Results

Activation of hypothalamic ATF4 by icv injection of adenovirus expressing ATF4 (Ad-ATF4) induces hepatic insulin resistance

To investigate the possible involvement of hypothalamic ATF4 in the regulation of insulin sensitivity, we icv 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 was demonstrated by the increased expression of Tribbles Homolog (TRB)3, one of the well known targets of ATF4 (24), in the hypothalamus, compared with Ad-GFP group (Figure 1A and 1B). Immunohistochemistry (IHC) 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 (Figure 1C).

The effects of icv injection of Ad-ATF4 was then examined. Although icv 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 Ad-GFP group (Figure 2A and 2B). Consistently, the HOMA-IR index was increased in these mice (Figure 2C). Icv injection of Ad-ATF4 significantly induced glucose intolerance and attenuated glucose clearance, as
demonstrated by glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs), respectively, compared with Ad-GFP group (Figure 2D and 2E, and S1A).

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 these 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 Ad-GFP group (Figure 2F).

**Inhibition of ATF4 in the hypothalamus by icv 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 icv injection of Ad-DN-ATF4, which is recognized as 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 Ad-GFP group (Figure 3A and 3B). Icv injection of Ad-DN-ATF4 had no effect on levels of blood glucose and serum insulin at fed state, however, it greatly decreased fasting serum insulin levels, though fasting blood glucose levels were unaffected, compared with
Ad-GFP group (Figure 3C and 3D). Consistently, the HOMA-IR index was also decreased significantly in these mice (Figure 3E). Glucose tolerance and insulin sensitivity were greatly improved by icv injection of Ad-DN-ATF4 compared with control group, as evaluated by GTTs and ITTs, respectively (Figure 3F and 3G, and S1B). Consistent with these changes, icv injection of Ad-DN-ATF4 also significantly enhanced insulin-stimulated phosphorylation of IR and AKT in the liver compared with Ad-GFP group (Figure 3H).

**Selective hepatic vagotomy reverses hypothalamic ATF4-induced insulin resistance in the liver**

Previous studies have shown that vagus nerve innervations is critical in mediating CNS regulating of hepatic glucose homeostasis (4; 6; 7), suggesting a possible involvement of 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 icv injection of Ad-ATF4 or Ad-GFP.

As expected, icv injection of Ad-ATF4 increased ATF4 and TRB3 protein levels in the hypothalamus compared with Ad-GFP group (Figure 4A). Consistent with previous results (Figure 1), icv injection of Ad-ATF4 had no effect on blood glucose and serum insulin levels at fed state, which were also not affected by hepatic vagotomy compared with control mice (Figure 4B and 4C). By contrast, increased fasting blood glucose levels by Ad-ATF4 were largely decreased by hepatic vagotomy
compared with sham surgery, though increased fasting insulin levels by Ad-ATF4 were not changed by hepatic vagotomy (Figure 4B and 4C). The increased HOMA-IR index by Ad-ATF4 was also significantly reduced by hepatic vagotomy (Figure 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 (Figure 4E-4G, and S1C).

**ATF4 stimulates hypothalamic mTOR/S6K1 phosphorylation *in vitro and in vivo***

Previous studies have shown that activation of 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 effects of knocking down ATF4 expression on 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 (Figure 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 (Figure 5B).
Based on these observations, we examined this relationship between ATF4 and S6K1 phosphorylation *in vivo* and similar results were obtained (Figure 5C and 5D).

**Icv injection of adenovirus expressing small hairpin (sh) RNA directed against the coding region of S6K1 (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 icv 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 S6K1 downstream target S6 in the hypothalamus in the presence or absence of icv injection of Ad-ATF4 (Figure 6A). Although no differences on fed blood glucose and serum insulin levels were observed among different groups, fasting blood glucose and serum insulin levels were increased significantly in Ad-ATF4 mice compared with Ad-GFP group, and Ad-shS6K1 prevented these upregulation (Fig. 6B and 6C). Consistent with these changes, the Ad-ATF4-mediated increased HOMA-IR index was also decreased by inhibition of S6K1 activity (Figure 6D). As mentioned above (Figure 1), icv injection of Ad-ATF4, in the presence of Ad-scramble, also impaired glucose tolerance and clearance compared with control mice co-injected with Ad-scramble and Ad-GFP (Figure 6E and 6F). Consistent with a role for S6K1 in mediating the effect of ATF4 on insulin sensitivity, icv injection of Ad-shS6K1 largely prevented Ad-ATF4-induced
impairment in glucose tolerance and clearance, and insulin signaling in the liver (Figure 6E-6G, and S1D). Ad-shS6K1 alone decreased fasting serum levels compared with control mice, HOMA-IR index was not significantly changed, which could be due to unchanged fasting blood glucose levels in these mice (Figure 6B-D). Except for this, Ad-shS6K1 alone treatment also improved insulin sensitivity (Figure 6A-G).

**Inhibition of ATF4 in the hypothalamus by icv 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 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 icv injection of Thapsigargin (TG), a classical ER stress-inducing chemical that has been extensively used (3), in mice icv injected with Ad-GFP or Ad-DN-ATF4.

Consistent with previous reports (3), icv injection of TG increased phosphorylation of PERK and eIf2α, well known as ER stress marker (16), as well as expression levels of ATF4 (Figure 7A). The possible involvement of ATF4 in mediating brain ER stress-induced hepatic insulin resistance was then investigated in mice icv injected with Ad-DN-ATF4 or Ad-GFP, followed by icv injection of TG. TG treatment had no effect on fed blood glucose and serum insulin levels in any group (Fig. 7B and C). By contrast, it significantly increased fasting blood glucose and
serum insulin levels, which were largely reversed by icv injection of Ad-DN-ATF4 (Figure 7B and 7C). The increased HOMA-IR index by TG treatment was also decreased in these mice (Figure 7D). 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 icv injection of Ad-DN-ATF4 (Figure 7E-7G, and S1E). Similar to those observed above (Figure 5), TG treatment-increased S6 phosphorylation was largely blocked by Ad-DN-ATF4 compared with control group (Figure 7H).

**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 icv 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 the 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 icv 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 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 resistance in various cell lines (30; 31) via directly phosphorylating several serine residues on insulin receptor substrate (IRS)1 (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 for 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 ameliorated 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, 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 hypothalamic ATF4 effect on hepatic insulin sensitivity, but also supports a role for hypothalamic S6K1 in regulating peripheral insulin sensitivity.

A variety of intracellular stresses have been recognized as primary pathogenic factors to insulin resistance (38), including ER stress, which initiates a set of intracellular responses that interfere the normal function of the ER (39-41). Increased ER stress has been observed in insulin-secreting pancreatic beta-cells (39) and additional insulin-responsive peripheral tissues (40; 41). Recent studies have shown that ER stress is also induced in the hypothalamus by over-nutrition (15) and contributes to insulin resistance directly via altered NFkB 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 current study, we used pharmacologic 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 a 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 (IRE1)/x-box-binding protein 1 (XBP1) and activating transcription factor 6 (ATF6) are also involved in 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 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 were prolonged as shown previously in other study (44).

In current study, mechanisms by which ATF4 regulates mTOR/S6K1 signaling is 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 ATF4 regulation of S6K1 activity in the hypothalamus. Consistent with these results, we found that AMPK phosphorylation was inhibited by hypothalamic ATF4 and ATF4-stimulated phosphorylation of mTOR and S6K1 were significantly blocked by constitutively active (CA)-AMPKα1 (46) in vitro (Figure S2). 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 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 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 make major contribution to the effects of hypothalamic ATF4 on hepatic insulin sensitivity, since food intake, body weight and fat mass were only increased about
8 %, 4 % and 8 %, respectively, by Ad-ATF4 (Figure S3), and insulin-stimulated phosphorylation of IR and AKT were also not detected earlier in muscle and WAT than in liver in Ad-ATF4 mice (Our 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 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. Specific neurons involved are currently under investigation.

In summary, the experiments described in this paper 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 (Figure 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.
Acknowledgments

This work was supported by grants from National Natural Science Foundation (81130076, 31271269, 81100615 and 30890043), the Ministry of Science and Technology of China (973 program 2010CB912502 and 2009CB919001; 2011ZX09307-302), 2010 Key Program of Clinical Research Center, INS, SIBS, CAS (CRC2010005), Key Program of Shanghai Scientific and Technological Innovation Action Plan (10JC1416900), the Knowledge Innovation Program of CAS (KSCX2-EW-R-09) and Chinese Academy of Sciences-funded project [2011KIP307]. Dr. Feifan Guo was also supported by the One Hundred Talents Program of CAS.

No potential conflicts of interest relevant to this article were reported.

Q.Z. researched data, wrote, reviewed, and edited the manuscript. J.Y. researched data, reviewed and edited the manuscript. Z.L. researched data. B.L. researched data and provided research material. T.X., F.X. and S.C. provided research material. F.G. contributed to discussion and wrote, reviewed, and edited the manuscript. F.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References:


19. Morton GJ, Gelling RW, Niswender KD, Morrison CD, Rhodes CJ, Schwartz MW: Leptin regulates insulin sensitivity via phosphatidylinositol-3-OH kinase signaling in medio basal...


45. Woods SC, Seeley RJ, Cota D: Regulation of food intake through hypothalamic signaling networks involving mTOR. Annual review of nutrition 2008;28:295-311


**Figure legends**

**Fig. 1** Icv injection of Ad-ATF4 increases ATF4 expression in the hypothalamus.
Mice received icv 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 ± SEMs shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiments 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 versus 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; PVN, paraventricular nucleus of hypothalamus; Arc, arcuate nucleus. Images shown are representative of several animals for each group.

**Fig. 2 Activation of hypothalamic ATF4 by icv injection of Ad-ATF4 induces hepatic insulin resistance.** Mice received icv 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 U/kg insulin stimulation for 3 min. Means ± SEMs shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiments 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 *versus* 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 icv injection of Ad-DN-ATF4 improves hepatic insulin sensitivity. Mice received icv 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 U/kg insulin stimulation for 3 min. Means ± SEMs shown are representative of at least two independent *in vivo* experiments, with the number of mice included in each group in each experiments indicated (n = 6 for each group). Statistical significance was calculated by two-tailed student *t*-test: *p <0.05 [for the effect of with *versus* 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).

**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 icv 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 U/kg insulin stimulation for 3 min. Means ± SEMs shown are representative of at least two independent in vivo experiments, with the number of mice included in each group in each experiments 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 Student-Newman-Keuls (SNK) test: *P < 0.05 [for the effect of any group versus without Ad-ATF4], and #P < 0.05 [for the effect of with versus without vagotomy in 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).

**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 icv 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 ± SEMs 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 experiments 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 versus 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).

**Fig 6. Inhibition of hypothalamic S6K1 by icv injection of Ad-shS6K1 reverses hypothalamic ATF4-induced hepatic insulin resistance.** Mice received icv 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 U/kg insulin stimulation for 3 min. Means ± SEMs shown are representative of at least two independent *in vivo* experiments, with the number of mice included in each group in each experiments 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 Student-Newman-Keuls (SNK) test: *P < 0.05 [for the effect of any group versus without Ad-ATF4], and #P < 0.05 [for the effect of with versus 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).

**Fig. 7 Inhibition of hypothalamic ATF4 reverses hepatic insulin resistance induced by acute brain ER stress.** Mice received icv injection of Ad-DN-ATF4 (+ Ad-DN-ATF4) or Ad-GFP (- Ad-DN-ATF4), followed by treatment with 1ug/d TG (+ TG) or without (- TG) for 3 consecutive days, prior to all measurements on day 7 post-adenoviral injection. Hepatic insulin signaling was examined before (- Ins) and after (+ Ins) 2 U/kg insulin stimulation for 3 min. Means ± SEMs shown are representative of at least two independent *in vivo* experiments, with the number of mice included in each group in each experiments 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 versus without TG treatment] in A, or by one-way ANOVA followed by the Student-Newman-Keuls (SNK) test: *P < 0.05 [for the effect of any group with versus without TG], and #P < 0.05 [for the effect of with versus 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 p-S6 relative to its total protein); (I) Working model.
Fig. 1

A

HYPO

Ad-ATF4

- +

ATF4

TRB3

actin

B

Relative mRNA (%)

0

300

200

100

ATF4 TRB3

C

Ad-ATF4

- + - +

4x

3v

Arc

10x

3v

PVN
**Fig. 3**

**A**

Ad-DN-ATF4

HYPO

- Ad-DN-ATF4

+ Ad-DN-ATF4

ATF4

TRB3

actin

Arbitrary Units

**B**

Relative mRNA (%)

ATF4

TRB3

**C**

Blood Glucose (mg/dl)

Fed

Fasting

**D**

Serum Insulin (ng/ml)

Fed

Fasting

**E**

HOMA-IR

**F**

GTT

Blood Glucose (mg/dl)

Time (min)

**G**

ITT

Blood Glucose (mg/dl)

Time (min)

**H**

Liver

- Ad-DN-ATF4

+ Ad-DN-ATF4

Ins

Ad-DN-ATF4

p-IR

t-IR

p-AKT

t-AKT

Arbitrary Units
**Figure 5**

**A**

Primary Culture

- DN-ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1
- ATF4
- actin

**Arbitrary Units**

- DN-ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1
- ATF4
- actin

**B**

Primary Culture

- ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1
- ATF4
- actin

**Arbitrary Units**

- ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1
- ATF4
- actin

**C**

HYPO

- Ad-DN-ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1

**Arbitrary Units**

- Ad-DN-ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1

**D**

HYPO

- Ad-ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1

**Arbitrary Units**

- Ad-ATF4
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1
Fig. 6

A. Western blot analysis showing the effects of Ad-ATF4 and Ad-shS6K1 on phosphorylation of various proteins. 

B. Graph showing blood glucose levels in fed and fasting conditions. 

C. Graph showing serum insulin levels in fed and fasting conditions. 

D. Graph showing HOMA-IR levels in fed and fasting conditions. 

E. Graph showing GTT results with different treatments. 

F. Graph showing ITT results with different treatments. 

G. Western blot analysis showing the effects of Ad-ATF4 and Ad-shS6K1 on phosphorylation of IR and AKT in liver tissue.
Diabetes

- TG - Ad-DN-ATF4
- TG - Ad-DN-ATF4
- TG + Ad-DN-ATF4

A

HYPO

<table>
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<tr>
<th>TG</th>
<th>-</th>
<th>+</th>
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</table>

- p-PERK
- t-PERK
- p-eif2α
- t-eif2α
- ATF4
- actin

B

Blood Glucose (mg/dl)

- fed
- fasting

C

Serum Insulin (ng/ml)

- fed
- fasting

D

HOMA-IR

- * |
- # |

E

GTT

Blood Glucose (mg/dl)

- * |
- # |

F

ITT

Blood Glucose (mg/dl)

- * |
- # |
G

<table>
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<tr>
<th>Ins</th>
<th>TG</th>
<th>Ad-DN-ATF4</th>
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Liver

- **p-IR**: arbitrary units
- **t-IR**: arbitrary units
- **p-AKT**: arbitrary units
- **t-AKT**: arbitrary units

H

- **TG Ad-DN-ATF4**
- **p-S6**: arbitrary units
- **t-S6**: arbitrary units

I

- **TG**: Hypothalamus
- **Hypothalamus**: ATF4 → S6K1
- **Liver**: p-IR ↓→ p-AKT ↓
- **Insulin resistance**: Vagus

**Fig. 7**
Plasmids encoding ATF4 (+ ATF4) or control vector (- ATF4), without (- CA-AMPK1) or with post-adenoviral injection. (B and C) Primary cultured hypothalamic neurons were transfected with Ad-ATF4, prior to examination of AMPK phosphorylation in the hypothalamus on day 7 post-adeno- viral injection. Supplementary Figure 2. ATF4 regulates mTOR/S6K1 signaling via AMPK. (A) Mice received icv injection of adenovirus expressing ATF4 (+ Ad-ATF4) or green fluorescent protein (- Ad-ATF4), prior to examination of AMPK phosphorylation in the hypothalamus on day 7 post-adeno- viral injection. (B and C) Primary cultured hypothalamic neurons were transfected with plasmids encoding ATF4 (+ ATF4) or control vector (- ATF4), without (- CA-AMPK1) or with constitutively active (CA)-AMPK1 (+ CA-AMPK1). Means ± SEMs shown are representative of
at least three independent *in vitro* experiments or at least two independent *in vivo* experiments, with the number of mice included in each group in each experiment indicated (n = 5 for each group *in vivo*, n = 6 for each group *in vitro*). Statistical significance was determined using the two-tailed Student’s *t*-test for A and B, or one-way ANOVA followed by the Student-Newman-Keuls (SNK) test for C: *p < 0.05 [for the effect of overexpression of ATF4 versus control group], and # *p < 0.05 [for the effect of with versus without CA-AMPK in ATF4 group]. (A) hypothalamic p-AMPK protein (top, western blot; bottom, quantitative measurements of p-AMPK protein relative to its total protein); (B and C) p-AMPK, p-mTOR, p-S6K1 and ATF4 protein in primary cultured hypothalamic neurons (left, western blot; right, quantitative measurements of p-AMPK, p-mTOR, p-S6K1 and ATF4 protein relative to their total protein or actin).

**Supplementary Figure 3.** Food intake, body weight and fat mass in mice icv injected with Ad-ATF4. Mice received icv injection of Ad-ATF4 (+ Ad-ATF4) or control green fluorescent protein (- Ad-ATF4), food intake was monitored daily, body weight and fat mass were measured on day 7 post-adenoviral injection. Means ± SEMs shown are representative of at least two independent *in vivo* experiments, with the number of mice included in each group in each experiments indicated (n = 7 for each group in food intake and WAT weight measurement; n = 6 for each group in body weight measurement). Statistical significance was determined using the two-tailed Student’s *t*-test: *p < 0.05 [for the effect of Ad-ATF4 versus control]. (A) Food intake; (B) Body weight change; (C) Fat mass.
**Fig. S2**

(A) HYPO

- Ad-ATF4
- p-AMPK
- t-AMPK

(B) Primary Culture

- ATF4
- p-AMPK
- t-AMPK

(C) Primary Culture

- ATF4
- CA-AMPK
- p-mTOR
- t-mTOR
- p-S6K1
- t-S6K1
- p-S6
- t-S6
- p-AMPK
- t-AMPK
- actin

**Bar Graphs**

- Arbitrary Units

- p-S6K1
- p-S6
- p-mTOR

- # # #
- * * *

- 0 50 100 150
- 0 100 150
- 0 50 100
- 0 100 150
- 0 100 200

- ATF4 - CA-AMPK
- ATF4 - CA-AMPK
- ATF4 + CA-AMPK
- ATF4 + CA-AMPK
Fig. S3

- Food intake (g/day)
- Body weight change (%)
- Abdominal fat mass / body weight (%)