differences in hepatic expression levels of IRS-1 protein between raptor-ΔC_T and control mice. Insulin-induced IRS-1 tyrosine phosphorylation was significantly increased in hepatic raptor-ΔC_T-overexpressing mice (Fig. 5*B*), whereas insulin-induced IRS-1 Ser^{307} and $Ser^{636/639}$ phosphorylation were markedly depressed in raptor-ΔC_T mice (Fig. 5, *C* and *D*). Moreover, we performed PI 3-kinase assays of the liver to investigate PI 3-kinase activity. Figure 6 presents insulin-induced tyrosine phosphorylation-associated PI 3-kinase activity and IRS-1-associated PI 3-kinase activity, both of which were increased approximately twofold compared with those of LacZ mice. Insulin-induced Akt Ser^{473} and Thr^{308} phosphorylations were markedly increased in raptor-ΔC_T mice (Fig. 7, *B* and *C*), as shown by immunoblotting of liver lysates with Akt and phospho-Akt Ser^{473} and Thr^{308} antibodies, but there was no difference between these mice in Akt protein expression (Fig. 7*A*). In addition, basal Akt Ser^{473} and Thr^{308} phosphorylations were also markedly increased in raptor-ΔC_T mice (Fig. 7, *B* and *C*).

DISCUSSION

Insulin resistance is induced by many factors, including obesity, high-fat diet, insufficient exercise, hypertension (13), and various hormones. Among these factors, obesity induced by excessive caloric intake is considered to be the most common and important factor leading to the occurrence of diabetes mellitus. In obese animals, PI 3-kinase activation via the association with IRS proteins is impaired, and increased serine phosphorylation in IRS-1 is reportedly involved in this impaired insulin-induced PI 3-kinase activation. Phosphorylation of serine residues of IRS-1 is also reportedly involved in IRS-1 degradation (1, 18, 25). To date, several serine/threonine kinases have been reported to phosphorylate serine residues of IRS-1.

IRS-1 phosphorylation mechanisms under insulin-resistant conditions can essentially be divided into two major categories. One involves adipocyte-derived factors such as TNFα, resistin, and free fatty acids, which activate JNK and/or ERK and thereby increase the serine phosphorylation of IRS-1. The other operates in response to intracellular nutrient conditions. The nutritional status of the cell directly regulates the AMPK/mTOR pathway independently of proteins secreted by adipocytes, and mTOR and S6K reportedly enhance phosphorylation

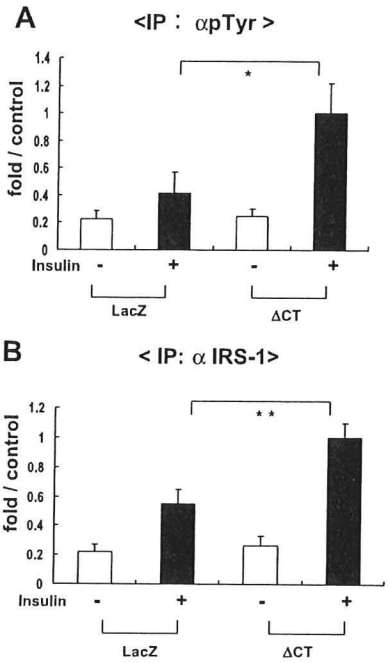

Fig. 6. Insulin-induced phosphatidylinositol (PI) 3-kinase activity in hepatic raptor-ΔC_T mice. For PI 3-kinase assay, supernatants containing equal amounts of protein were immunoprecipitated for 2 h at 4°C with anti-IRS-1 or phosphorylated tyrosine antibody and protein A- or G-Sepharose. PI 3-kinase activities in the immunoprecipitates were assayed. *A* and *B*: insulin-induced, tyrosine phosphorylation-associated PI 3-kinase activity and IRS-1-associated PI 3-kinase activity were both increased to approximately double those of LacZ mice. LacZ: $n = 8$ (insulin$^+$: $n = 4$; insulin$^-$: $n = 4$); ΔC_T: $n = 8$ (insulin$^+$: $n = 4$; insulin$^-$: $n = 4$). $^*P < 0.05$; $^{**}P < 0.01$.

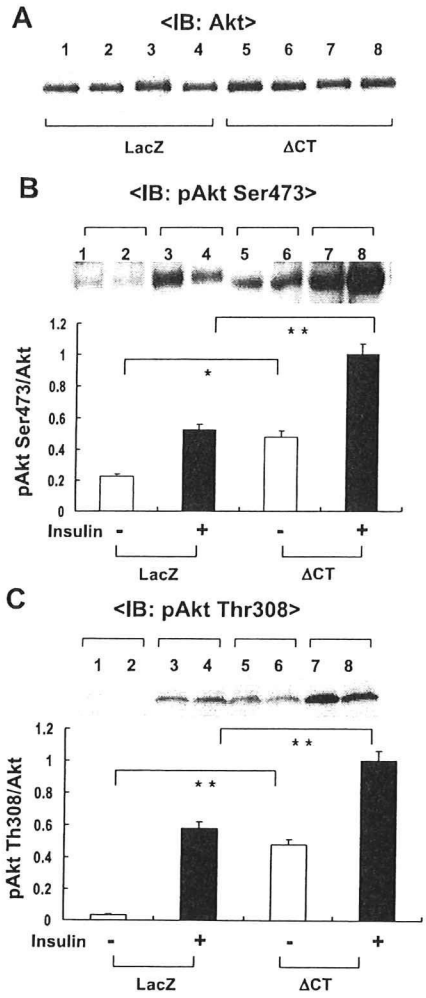

Fig. 7. Insulin-induced Akt phosphorylation in hepatic raptor-ΔC_T mice. Liver lysates were immunoblotted with Akt and phospho-Akt Ser^{473} and Thr^{308} antibody. *A*: there was no difference in Akt protein expression levels between these mice. *B* and *C*: basal Akt Ser^{473} and Thr^{308} phosphorylation as well as insulin-induced Akt Ser^{473} and Thr^{308} phosphorylation were also markedly increased in raptor-ΔC_T mice. LacZ: $n = 8$ (insulin$^+$: $n = 4$; insulin$^-$: $n = 4$); ΔC_T: $n = 8$ (insulin$^+$: $n = 4$; insulin$^-$: $n = 4$). $^*P < 0.05$; $^{**}P < 0.01$.

of serine residues of IRS-1 (3, 5, 16). Although S6K1-deficient mice were shown to be resistant to age- and diet-induced obesity and insulin resistance (26), we investigated the acute effect of transient inhibition of raptor on the impaired insulin signaling and glucose intolerance of K/KAy mice with genetic obesity-associated insulin resistance. In the K/KAy mice, one of the obese rodent models, IRS-1 Ser^{307} and IRS-1 $Ser^{636/639}$ phosphorylations are elevated (26).

Raptor contains a highly conserved amino-terminal domain, followed by several HEAT repeats and seven carboxy-terminal WD40 repeats (4), and acts as an adaptor to recruit substrates $p70^{S6k}$ and 4E-BP1 to mTOR (2, 12, 23). The domains in raptor and mTOR that interact with each other have been clearly demonstrated and suggest multiple contact sites between these two proteins (4, 10), in contrast with the selective binding of $p70^{S6k}$ to the NH_2-terminal portion of raptor (12). We were unable to detect the associations of raptor and COOH-terminally deleted raptor (raptor-ΔC_T) with endogenous S6K (data not shown). However, it was demonstrated that raptor-ΔC_T binds to a far smaller amount of mTOR but not to IRS-1, whereas wild-type raptor binds to both. Indeed, IRS-1 phosphorylation at $Ser^{636/639}$ was markedly decreased by raptor-ΔC_T overexpression. These findings suggest that raptor-ΔC_T functions as a dominant negative protein for mTOR/S6K or mTOR/IRS-1 signaling.

Interestingly, we found that 4E-BP1 phosphorylations of both $Thr^{37/46}$ and Thr^{70} in the liver were significantly increased by raptor-ΔC_T overexpression. Thus, the inhibitory effect of raptor-ΔC_T is specific for S6K. This result was unexpected, but it is hoped that it will provide useful information regarding how the raptor-mTOR complex recognizes individual downstream molecules. We speculate that S6K, but not 4E-BP1, preferentially associates with raptor-ΔC_T to full-length raptor. If so, raptor-ΔC_T overexpression would inhibit S6K binding, but not that of 4E-BP1, with the mTOR/raptor complex. It is also possible that some unidentified molecule is required for this association between S6K and the raptor-mTOR complex and that raptor-ΔC_T binds to this as yet unknown molecule. In this case, S6K cannot bind the mTOR complex in the raptor-ΔC_T-overexpressing cells, whereas 4E-BP1 phosphorylated is unaffected. Further study is necessary to resolve this issue.

In the present study, hepatic overexpression of raptor-ΔC_T strongly inhibited insulin-induced $p70^{S6k}$ activation and improved glucose intolerance and hyperinsulinemia. Importantly, Akt phosphorylation was markedly enhanced not only under insulin-stimulated but also basal conditions. Decreased IRS-1 Ser^{307} and $Ser^{636/639}$ phosphorylations and the resulting increases in tyrosine phosphorylation of IRS-1 and subsequent PI 3-kinase activity can account for the increased Akt phosphorylation under insulin-stimulated conditions. However, this may not fully explain the mechanism leading to markedly increased basal Akt phosphorylation since basal PI 3-kinase activity was not altered by raptor-ΔC_T. Thus, it is possible that other mechanisms, such as increased PDK and/or rictor activity, or even suppression of Akt dephosphorylation, are involved in the increased basal Akt phosphorylation. Indeed, it has been reported (22) that raptor-mTOR and rictor-mTOR complexes regulate Akt phosphorylation in a reverse manner. Further study is necessary to clarify whether suppression of the raptor-mTOR complex via overexpression of raptor-ΔC_T leads to elevated rictor-mTOR activity or suppressed Akt dephosphorylation.

In summary, we demonstrated that hepatic $p70^{S6k}$ inhibition in diabetic mice improves glucose tolerance by enhancing both basal and insulin-stimulated Akt phosphorylations. Although further experiments are needed to clarify the molecular mechanisms of increased basal Akt phosphorylation, our results suggest that mTORC1 inhibition is a potential treatment strategy for obesity-related insulin resistance.

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Inter-organ metabolic communication involved in energy homeostasis: Potential therapeutic targets for obesity and metabolic syndrome

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Abstract

The global rate of obesity is rising alarmingly, exerting a major adverse impact on human health by increasing the prevalences of disorders, such as diabetes, hypertension and heart disease. To maintain systemic energy homeostasis, metabolic information must be communicated among organs/tissues. Obesity-related disorders can be thought of as resulting from dysregulation of this vital inter-tissue communication. Remarkable advances in obesity research during this decade have shown humoral factors manufactured and secreted by adipose tissue (adipocytokines) to be of great importance. In addition to these humoral factors, such as nutrients (glucose, fatty acids and amino acids) and hormones (insulin, adipocytokines and so on), the functional significance of the autonomic nervous system has recently attracted research attention. Autonomic nerves are essential components of the endogenous system for maintaining energy homeostasis, making them potential therapeutic targets for obesity-related disorders. This review focuses on the therapeutic possibilities of targeting inter-organ communication systems.

Keywords: Obesity; Metabolic syndrome; Inter-organ communication; Energy homeostasis; Autonomic nervous system; Central nervous system

Contents

1. Introduction

The incidence of obesity, a major risk factor for numerous disorders, including diabetes, hypertension and heart disease, is rising at an alarming rate in much of the world (Flier, 2004). Body weight is generally accepted to be determined by the balance between energy intake and expenditure. Normal weight individuals are reportedly protected against the expansion of body fat stores induced by overfeeding (Leibel et al., 1995), indicating the existence of biological mechanisms which protect against weight gain, as well as weight loss,

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at least in normal weight individuals. Energy homeostasis, maintained by multiple mechanisms, involves collecting information on systemic nutritional status and responding appropriately, both behaviorally and metabolically, to changes in fuel availability. Humoral factors, including insulin and adipocytokines, are known to be very important for this inter-organ/tissue communication. In addition, we and other investigators have recently demonstrated the autonomic nervous system to have a key role in transmitting metabolic information. Employing these systems, the brain gathers information on peripheral metabolic status, processes it, and then sends signals which regulate metabolism in the periphery. The hypothalamus, in particular, is a primary site of convergence and integration for redundant energy status signaling, which encompasses both central and peripheral neural inputs as well as hormonal and nutritional factors. These inter-tissue communication pathways are summarized in (Fig. 1; Yamada & Katagiri, 2007).

All but the most severe obesity cases can be successfully managed, solely through lifestyle modifications, i.e., improvements in diet and promotion of greater physical activity. However, low compliance with these strategies has generated interest in alternative effective therapies, including gastrointestinal bypass surgery (efficacious and long-lasting, but limited in use because of associated risks and costs) and pharmacological interventions. The market for safe and efficacious drugs is therefore potentially enormous, though the value of currently approved therapies does not reflect this potential, due to the limited efficacies and side-effect profiles of these treatments. This review summarizes our current understanding of the roles of inter-tissue communication in energy homeostasis and suggests potential therapeutic targets for obesity and the metabolic syndrome.

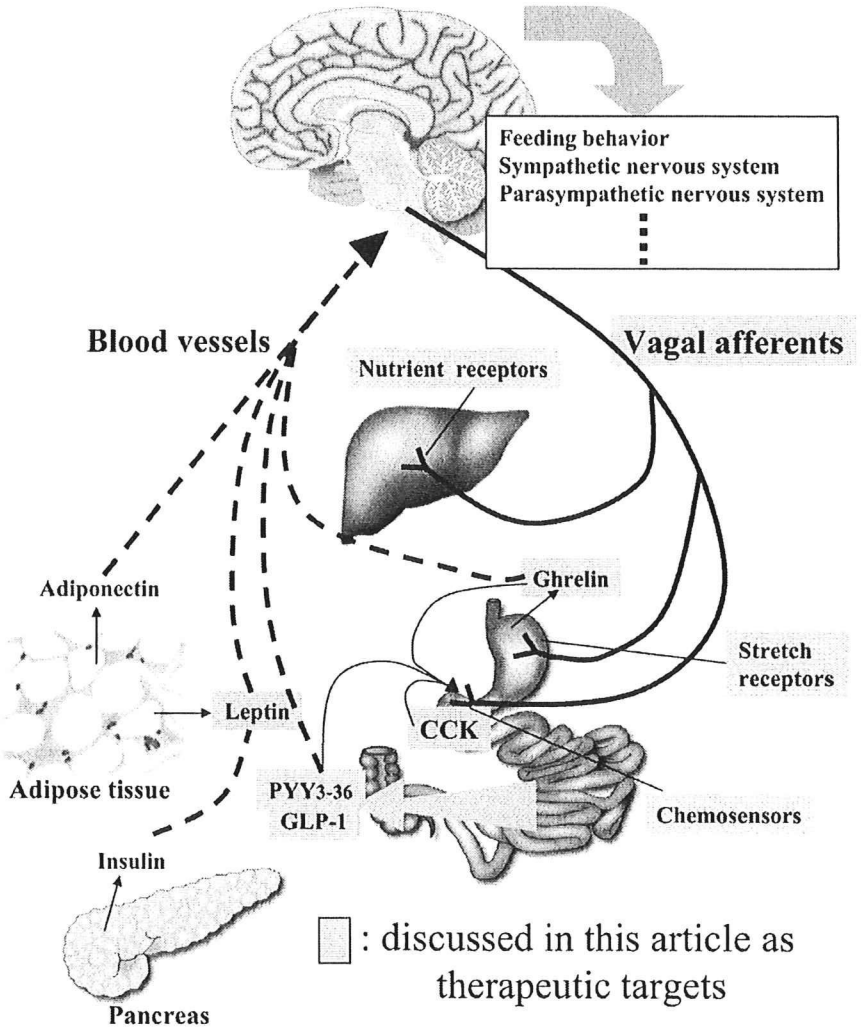

Fig. 1. Schematic presentation of intertissue communication (quoted with slight modification from Yamada & Katagiri, 2007). The brain receives various forms of metabolic information from peripheral organs/tissues through humoral and neuronal pathways. These inputs are probably integrated and processed in the brain, leading to appropriate systemic responses. Several signals, as therapeutic targets, are discussed in this article.

2. Neuroendocrine regulation of body weight and therapeutic implications for obesity

2.1. Brain inputs — humoral factors

2.1.1. Nutrients

The brain senses and then responds to nutrient-related signals arising from changes in intracellular energy contents or in either the availability or metabolism of substrates, such as free fatty acids. Some of these signals are generated in response to decreases in substrates, while others represent responses to nutrient excesses.

2.1.1.1. Glucose. In addition to serving as the primary fuel source for the brain, glucose metabolism in a subset of neurons (so-called "glucose-responsive" and "glucose-sensitive" neurons) generates signals that regulate membrane potential and neuronal firing. In glucose-responsive neurons, the molecular mechanism underlying this glucose effect resembles that, whereby glucose stimulates insulin secretion from pancreatic β cells, resulting in increased firing rates (Rowe et al., 1996). Such neurons have been characterized mainly in the ventromedial hypothalamic (VMH) nucleus and the arcuate (ARC) nucleus (Levin et al., 2004). Glucose metabolism in these cells activates KATP channels, allowing K^+ efflux, and thereby hyperpolarize the cells. KATP channel activity is a key step in converting metabolic changes into the electrical activity of ARC and VMH neurons (Wang et al., 2004). Interestingly, KATP channel activation by glucose in ARC glucose-responsive neurons is attenuated by insulin and leptin via a phosphatidylinositol 3OH kinase (PI3K)-dependent mechanism (Spanswick et al., 2000). Neither the underlying mechanism nor the extent to which these glucose-sensing neurons contribute to the actions of insulin and leptin, in neuroendocrine control of energy homeostasis, has as yet been determined.

In contrast, in glucose-sensitive neurons, firing is suppressed by glucose (Levin et al., 1999). Membrane potential effects mediated by tandem-pore K^+ (K_{2P}) channels were recently reported to be involved in glucose-induced inhibition of orexin neurons, a subset of glucose-sensitive neurons (Burdakov et al., 2006). In these neurons (in this case), glucose metabolism to ATP is not required. Thus, several types of potassium channels, including KATP and K_{2P} channels, are likely to play important roles in glucose sensing in a variety of neurons. However, the molecular mechanisms by which glucose suppresses firing in glucose-sensitive neurons is still largely unknown.

2.1.1.2. Free fatty acids. The access of circulating free fatty acids to cerebrospinal fluids is generally proportional to the plasma fatty acid concentration (Miller et al., 1987; Rapoport, 1996), indicating the brain to possibly acquire information about the peripheral metabolic state via cerebrospinal fluid fatty acid levels. Fatty acid-sensitive neurons have been identified in the hypothalamus. As an example, an *in vitro* patch clamp study (Wang et al., 2006) demonstrated 13% of arcuate neurons to show increased electrical activity, while 6% showed decreased activity, when oleic acid was applied. Several recent studies have examined the role of cerebrospinal fluid fatty acids in energy metabolism. Intracerebroventricular (i.c.v.) administration of oleic acid reportedly inhibits both hepatic glucose production and food intake (Obici et al., 2002). In addition, hypothalamic inhibition of carnitine/palmitoyl-coenzyme A transferase-1 (CPT-1), an important mitochondrial enzyme for transfer of long-chain fatty acyl-coenzyme A (LCFA-CoA) into mitochondria, decreases food intake and suppresses endogenous glucose production (EGP) in the liver (Obici et al., 2003). Efferent vagal nerve signals from the brain to the liver are also reportedly involved in hepatic gluconeogenesis in these experimental settings (Pocai et al., 2005a, 2005b). Hu et al. found that central administration of C75, a potent fatty acid synthase (FAS) inhibitor, decreased food intake (Hu et al., 2003). Since FAS inhibition increases malonyl-CoA and thereby suppresses CPT1 activity, LCFA-CoA in hypothalamic neurons would appear to be increased. These results, taken together, indicate the cytoplasmic LCFA-CoA concentration in hypothalamic neurons to play an important role in energy homeostasis. Further studies are needed to clarify the mechanisms regulating the neuronal LCFA-CoA content, its relationship to plasma free fatty acid (FFA) levels and the intracellular mechanism whereby a change in the LCFA-CoA content alters neuronal function.

2.1.1.3. Amino acids. Amino acids also apparently transmit energy status information from the periphery. Amino acids are reportedly transported across the blood–brain barrier (Choi et al., 2001). Therefore, the amino acid levels in cerebrospinal fluids appear to reflect those in peripheral blood. Centrally administered leucine increases hypothalamic mammalian target of rapamysin (mTOR) activity, thereby decreasing both food intake and body weight (Cota et al., 2006). mTOR is a highly conserved serine/threonine kinase found in organisms from yeast to mammals. mTOR activity sensitive to branched chain amino acid levels, especially that of L-leucine (Proud, 2002; Meijer & Dubbelhuis, 2004). Thus, mTOR is known to be among the energy sensors for amino acids conserved throughout evolution and, in mammals, hypothalamic mTOR signaling apparently plays an important role in regulating systemic energy metabolism. Leptin increases hypothalamic mTOR activity, and inhibition of mTOR signaling suppresses leptin's anorectic effect (Cota et al., 2006). However, further studies are needed to fully clarify mTOR's role in energy homeostasis.

2.1.2. Hormonal signals

2.1.2.1. Insulin. Insulin, produced by pancreatic β cells, is the master metabolic switch between fed and fasted states, mediating metabolic fuel disposition and use. Some investigators speculate that insulin itself might signal fuel status to the brain, but the actual mechanisms by which insulin would exert such effects have long eluded clarification.

An electrophysiological study showed inhibitors of PI3K to block the capacity of insulin to hyperpolarize hypothalamic "glucose-responsive" neurons (Spanswick et al., 2000). A subsequent in vivo study showed i.c.v. infusion of PI3K inhibitors to effectively prevent insulin-induced anorexia (Niswender et al., 2003). Furthermore, activation of insulin signaling in the ARC

alone, in the absence of elevated systemic insulin, is sufficient to decrease not only food intake but also blood glucose levels, by markedly inhibiting EGP in the liver (Plum et al., 2006; Prodi & Obici, 2006). A recent study revealed the central effects of insulin on this hepatic EGP suppression to be mediated by KATP channel activation through the insulin receptor (IR)–insulin receptor substrate 2 (IRS2)–PI3K pathway in the ARC (Pocai et al., 2005a, 2005b). Thus, intracellular insulin signal transduction in the brain, particularly in the hypothalamic ARC nucleus, plays an important role in regulating food intake, as well as in systemic glucose metabolism.

2.1.2.2. Leptin. Leptin was identified by positional cloning using the *ob/ob* mouse model (Zhang et al., 1994) as a key molecule in the regulation of both body weight and energy balance. Leptin is produced mainly by adipocytes in proportion to fat stores. Adipocyte leptin expression is transcriptionally regulated, being determined mainly by adipocyte size. Adequate leptin levels communicate the status of energy stores in white adipose tissue (WAT) to the central nervous system (especially the hypothalamus), suppressing food intake and permitting energy expenditure via sympathetic stimulation of several tissues (Haynes et al., 1997; Friedman & Halaas, 1998). As an example, when energy stores increase, the energy balance is negatively regulated by decreased food intake and increased energy expenditure (Friedman & Halaas, 1998). Leptin binds to the leptin receptor Ob-Rb in the hypothalamus, thereby activating the JAK-STAT (Bjorbaek et al., 1997; Bates & Myers, 2004) and IRS2-PI3K (Niswender et al., 2001) pathways. Leptin also suppresses hypothalamic AMPK activity and thus reduces food intake (Minokoshi et al., 2004). As described above, leptin also activates mTOR signaling in the hypothalamus. Thus, there appear to be complicated interactions among the (at least) 4 pathways, JAK-STAT, IRS2-PI3K, AMPK and mTOR, involved in leptin signaling.

In most individuals with ordinary obesity, circulating leptin is elevated, but the body does not adequately respond to higher leptin levels by reducing food intake. This lack of responsiveness to leptin in most forms of obesity raises the possibility that obesity is a state of relative leptin resistance. Leptin resistance is thought to be an important mechanism for maintaining the obese state.

2.2. Brain inputs — afferent nerve signals

2.2.1. Innervation

2.2.1.1. Intra-abdominal innervation without white adipose tissues. Innervation of intra-abdominal tissues warrants an explanation. Intra-abdominal tissues are innervated by both splanchnic (sympathetic) and vagal (parasympathetic) nerves. These nerve bundles consist of both efferent and afferent fibers. Detailed fiber count studies have revealed abdominal vagal and splanchnic nerves to be comprised of approximately 75% and 50% afferent fibers, respectively. Vagal afferents respond to specific chemical stimuli, the degree of physiological gut distention and nutrients, whereas splanchnic afferents carry information about noxious stimuli (Badman & Flier, 2005).

2.2.1.2. Innervation of intra-abdominal adipose tissues. WAT is also innervated by both efferent and afferent nerve fibers. Numerous reports have described the important metabolic roles, including lipolysis and β oxidation (Shimazu, 1981; Bartness & Bamshad, 1998; Imai et al., 2006), of efferent sympathetic fibers. Efferent parasympathetic innervation of WAT is controversial (Kreier et al., 2002; Giordano et al., 2006). On the other hand, afferent nerves from WAT have been demonstrated by several methods. Sensory innervation of WAT was directly demonstrated using a neuroanatomical approach with application of an anterograde tract tracer, True Blue, to WAT, resulting in labeling of neurons in rat dorsal root ganglia (Fishman & Dark, 1987). More recently, afferent innervation of epididymal WAT was demonstrated by another group using the pseudorabies virus as a retrograde neuronal tracer (Kreier et al., 2006).

2.2.2. Signals transmitted by afferent autonomic nerve fibers

2.2.2.1. Signals from the gut. Many peptides are synthesized and released by the gastrointestinal tract. Several of these peptides, such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and ghrelin, have been shown to modulate eating behaviors, (Woods & Gibbs, 1989; Stanley et al., 2004; Woods, 2004). Several of these peptides have direct access to brain regions involved in regulating food intake, such as the ARC of the hypothalamus and the area postrema, via the circulation. These peptides also function outside the CNS, influencing the activities of neurons, e.g., the vagal afferent nerve which projects to the nucleus of the solitary tract (NTS) in the brain stem. Further research is needed to determine the weighting and integration of each of these different signals.

2.2.2.1.1. CCK. CCK, produced by mucosal enteroendocrine cells of the duodenum and jejunum, is secreted in response to the presence of food in the gut lumen. Satiating effects of CCK have been confirmed based on the carboxy-terminal octapeptide of CCK reducing meal size and duration (Pi-Sunyer et al., 1982). Pharmacologic and genetic experiments have yielded evidence that the CCK1 receptor mediates CCK-induced satiation (Moran et al., 1998; Kopin et al., 1999). Sulfated CCK, which preferentially binds to CCK1R on vagal afferent neurons, signals satiety to the brain; this explains why vagotomy inhibits the anorectic effect of CCK (Smith et al., 1981). However, CCK1R is also expressed in both the hindbrain and the hypothalamus. Lesioning the hindbrain area postrema attenuates CCK-induced satiation (Edwards et al., 1986) and CCK microinjections into several hypothalamic nuclei lead to decreased food intakes (Blevins et al., 2000). Collectively, these observations suggest that CCK might relay satiation signals to the brain both directly and indirectly.

Continuously administering intraperitoneal CCK to rats results in reduced meal sizes, but this reduction is offset by increased meal frequency, such that there is no effect on body weight (West et al., 1984). In human subjects, food intake and gastric emptying were acutely reduced by CCK infusions (Muurahainen et al., 1988), but these anorectic effects disappeared after only 24 hr of continuous infusion (Crawley & Beinfeld, 1983). Therefore, although CCK clearly plays a role in terminating individual

meals, it appears to have little impact on long-term body weight regulation and thus seems unlikely to be an antiobesity drug target.

2.2.2.1.2. PYY3-36. The secretion of PYY3-36 from enteroendocrine L cells is triggered by luminal nutrients. Sugars activate L cells via the closure of ATP-sensitive potassium channels, which in turn leads to depolarization of the L cells via a mechanism analogous to insulin secretion from β cells (Reimann & Gribble, 2002; Gribble et al., 2003). PYY1-36 binds to all known Y receptors with similar affinities. In contrast, most circulating PYY immunoreactivity is in the amino-terminally truncated form, PYY3-36, which preferentially binds to Y2 receptors (Y2R). In the hypothalamus, Y2R is a presynaptic autoinhibitory receptor on orexigenic neurons expressing both NPY and agouti-related protein (AgRP), known as NPY/AgRP neurons. This has lead to the proposal that circulating PYY3-36 reduces food intake by inhibiting NPY/AgRP neurons through Y2R, thereby activating anorectic melanocortin-producing cells, which are inhibited by NPY/AgRP neurons (Batterham et al., 2002). Consistent with this model, the anorectic effects of PYY3-36 can be abolished by either pharmacologic or genetic ablation of Y2R (Batterham et al., 2002; Scott et al., 2005; Talsania et al., 2005). Though these lines of evidence support a hypothalamic mechanism of action for peripherally administered PYY3-36, Y2R is also expressed by vagal-afferent terminals (Koda et al., 2005), and some investigators have speculated that vagal mediation also exists. Supporting this hypothesis, the anorectic effects and arcuate neuronal activation induced by peripheral PYY3-36 were demonstrated to be eliminated by either subdiaphragmatic vagotomy or transection of hindbrain-hypothalamic pathways (Abbott et al., 2005; Koda et al., 2005). Assessed collectively, these observations suggest that PYY3-36 might also relay satiation signals, both direct and indirect, to the brain.

Therapeutic possibilities. Peripheral administration of PYY3-36 reduces food intake, and thereby body weight, in rodents, and these effects may be even more robust in primates (Moran et al., 2005), apparently due to activation of autoinhibitory Y2R on hypothalamic orexigenic NPY/AgRP neurons. In addition, recent evidence indicates vagal mediation of one component of PYY3-36-induced anorexia as described above. Intravenous infusion of a single dose (Batterham et al., 2003), as well as graded infusions (Degen et al., 2005) of PYY3-36, reportedly reduce appetite and food consumption by >30% in lean and obese subjects, although several investigators have encountered difficulties in attempting to reproduce these effects (Boggiano et al., 2005). It is noteworthy that obese subjects show normal sensitivity to the anorectic effects of PYY3-36, and circulating PYY levels are not elevated in the obese, in contrast to those of leptin (Batterham et al., 2003). The injectable PYY3-36 analogue AC-162352 was tested in phase I studies, with limited success due to nausea (Halford, 2006). In a phase I clinical trial as a nasally administered obesity treatment, on the other hand, PYY3-36 was both safe and well tolerated, and there was evidence of reduced caloric intake, appetite moderation and a tendency for weight loss in human subjects (Halford, 2006).

2.2.2.1.3. Glucagon-like peptide-1. GLP-1 is produced mainly by L cells located in the distal small intestine and colon, where it is colocalized with PYY. Ingested nutrients, especially fats and carbohydrates, stimulate GLP-1 secretion by indirect, duodenally activated neurohumoral mechanisms, as well as via direct contact within the distal intestine (Brubaker & Anini, 2003). The 2 equipotent bioactive forms, GLP-17-36 amide and GLP-17-37, are rapidly inactivated in the circulation by dipeptidyl peptidase-IV (DPP-IV; Orskov et al., 1993). GLP-1 has been shown to suppress food intake in several species (Turton et al., 1996; Donahey et al., 1998), including humans (Verdich et al., 2001). The mechanisms underlying GLP-1-induced anorexia are not fully understood but are believed to involve vagal and possibly direct central pathways. The GLP-1 receptor is expressed by organs/tissues, including the gut, pancreas, brainstem, hypothalamus and vagal-afferent nerves (Drucker, 2006). The anorectic effects of peripheral GLP-1 administration were shown to be abolished by vagotomy (Abbott et al., 2005; Talsania et al., 2005). Thus, peripheral GLP-1 also signals satiety to the brain via the vagal afferent pathway.

Therapeutic possibilities. Chronic subcutaneous GLP-1 administration for 6 weeks to obese subjects with type 2 diabetes led to a 1.9-kg body weight loss, on average (Zander et al., 2002). Given that the native GLP-1 peptide undergoes rapid enzymatic inactivation, DPP-IV-resistant GLP-1 analogues have attracted considerable attention as potential treatments for type 2 diabetes complicated by obesity. Exendin-4 is a naturally occurring 39 amino acid GLP-1 receptor agonist originally isolated from the venom of the *Heloderma suspectum* lizard (Eng et al., 1992). Exendin-4 has a glycine residue, which confers resistance to cleavage by DPP-IV, at position 2. In clinical trials, twice-daily subcutaneous administration of exendin-4 in patients with type 2 diabetes produced a dose-dependent weight loss of 1.8 kg during a period of 28 days (Poon et al., 2005) and 2.8 kg over 30 weeks (DeFronzo et al., 2005). Thus, in addition to promoting insulin secretion (incretin effects), the anorectic effects of GLP-1 agonists have attracted attention as possible diabetes treatments. This is because the improvements in glycemic control achieved with other oral glucose-lowering drugs typically promote weight gain.

2.2.2.1.4. Ghrelin. Ghrelin, a peptide recently found to be produced by the stomach, acts on a previously identified orphan receptor (growth hormone [GH] secretagogue receptor), the activation of which in the hypothalamus triggers the pituitary gland to release GH (Kojima et al., 1999). Ghrelin increases food intake in diverse species (Tschop et al., 2000), including humans (Wren et al., 2001). Date et al. (2002) reported gastric vagal afferent blockade to abolish ghrelin-induced feeding increases, GH secretion and the activations of NPY- and GH-releasing hormone (GHRH)-producing neurons. The ghrelin receptor is also expressed in vagal afferent terminals, and ghrelin suppresses vagal afferent firing. These findings, taken together, indicate gastric vagal afferent involvement in conveying signals regarding starvation, as well from the gut to the brain.

Therapeutic possibilities. In humans, feelings of hunger and food intake are both increased by either intravenous infusion or subcutaneous injection of ghrelin (Kojima & Kangawa, 2005). Therefore, blocking ghrelin signaling with ghrelin receptor antagonists has attracted interest as a possible strategy for preventing obesity. A ghrelin receptor antagonist reportedly

reduced food intake in fasted mice and an RNA Spiegelmer (an L-oligonucleotide designed to bind specifically to a particular molecule) inhibited ghrelin action both in vitro and in vivo (Cummings, 2006). It was recently demonstrated that vaccinating rats against ghrelin can suppress weight gain (Zorrilla et al., 2006). However, in obese individuals,ghrelin levels are low but rise in response to weight loss (Kojima & Kangawa, 2005). This is apparently part of a compensatory response that promotes weight regain. Therefore, the most clinically useful application of ghrelin receptor blockade might be in the prevention of rebound after weight reduction, which has been achieved by other means, rather than for initiating weight reduction de novo.

2.2.2.2. Signals from the liver

2.2.2.2.1. Hepatoportal glucose sensor. Sensor of short-term alterations in energy status. Blood glucose levels rise postprandially and decrease while fasting. Therefore, blood glucose concentrations reflect short-term energy status alterations. Glucose absorbed from the gut enters the portal vein, thereby reaching the liver directly. Thus, given its anatomical location, it is reasonable to assume that the liver functions as a glucose sensor. The hepatoportal glucose sensor is as yet incompletely defined, but reportedly consists of several components including GLUT2 (Burcelin et al., 2000a), as well as the GLP-1 receptor (Burcelin et al., 2001). Glucose entry into the hepatoportal vein triggers the activation of glucose sensors (Hevener et al., 1997), which can induce anorexia (Russek, 1963, 1970) and stimulate glucose uptake by the liver (Gardemann et al., 1986; Cardin et al., 1999), muscle, heart and brown adipose tissue (Burcelin et al., 2000b). Raising the portal vein glucose concentration decreases vagal afferent discharges reaching the NTS nuclei (Thorens & Larsen, 2004), indicating signals regarding portal glucose elevation to be carried along afferent vagal pathways. Hypoglycemic signals from the hepatoportal system, in contrast, involve splanchnic afferents. Reportedly, a counter-regulatory response to moderate systemic hypoglycemia, i.e., sympathetic efferent activation, is attenuated by clamping the liver at euglycemic levels and is blocked when splanchnic (but not vagal) afferents from the hepatic portal structure are interrupted (Donovan et al., 1991; Fujita & Donovan, 2005).

These observations, when considered collectively, indicate that the afferent autonomic nervous system, including both vagal and splanchnic nerves, from the hepatoportal circulation plays important roles in conveying information about peripheral glucose levels to the brain.

2.2.2.2.2. Peroxisome proliferator-activated receptors. Sensor of long-term alterations in energy status. Lipid mediators have key roles in metabolic control, and the peroxisome proliferator-activated receptor (PPAR) have emerged as the master transcriptional regulators of long-term lipid and carbohydrate metabolism (Desvergne et al., 2006). Saturated and unsaturated long-chain fatty acids and their eicosanoid derivatives are natural activators of this important subclass of nuclear receptors (Feige et al., 2006). Studies using mice with tissue-specific knockout of PPARγ have shown these receptors, in a number of organs, to function as a sensor of long-term energy status alterations. Notably, liver-specific disruption of PPARγ in ob/ob mice prevented hepatic steatosis, although a gradual increase in peripheral adiposity as well as decreases in the insulin sensitivities of muscle and adipose tissue were observed (Matsusue et al., 2003). In addition, hepatic expression of PPARγ, especially that of PPARγ2, is functionally enhanced in a number of obesity models (Chao et al., 2000; Rahimian et al., 2001). Therefore, hepatic PPARγ appears to play important roles not only in hepatic lipid storage but also in the regulation of both peripheral lipid metabolism and insulin sensitivity. The mechanism underlying this inter-organ/tissue communication between the liver and peripheral tissues, including muscle and fat, was recently revealed to involve autonomic nerve circuits (Uno et al., 2006).

The roles of hepatic PPARγ2 in peripheral metabolism were confirmed experimentally. Adenovirus-mediated PPARγ2 expression in the liver was shown to acutely induce severe hepatic steatosis, while peripheral adiposity was greatly reduced due to enhanced lipolysis. Systemic metabolic rates rose, such peripheral insulin sensitivity, and glucose tolerance showed marked improvements. These remote effects were attributable to increased sympathetic outflow into muscle and adipose tissues. Selective hepatic branch vagotomy significantly reversed both the peripheral adiposity reduction and the enhanced energy expenditure. Furthermore, pharmacological deafferentation of the vagus blocked the hepatic PPARγ2 expression-induced decrease in WAT weights. These findings indicate that hepatic PPARγ2 expression and/or hepatic lipid accumulation triggers the communication of metabolic information to the brain via afferent vagal nerve fibers, leading to antiobesity and antiinsulin-resistant effects in both muscle and adipose tissue (Uno et al., 2006).

Lipid storage in the liver changes dynamically according to the systemic energy balance and is known to be associated with several features of the metabolic syndrome. The liver may convey information regarding excess long-term energy storage to the central nervous system via the afferent vagus. This neuronal system is likely to underlie so-called chronic "adaptive thermogenesis," protecting the organism against metabolic perturbation induced by excessive energy storage (Fig. 2). The brain receives information regarding this excess energy storage, via leptin from adipose tissues as well as via the afferent vagus from the liver, activates the sympathetic nervous system to enhance energy expenditure and lipolysis, and thereby maintains energy homeostasis (Uno et al., 2006). A similar autonomic nerve circuit was recently shown to play an essential role in the development of glucocorticoid-induced insulin resistance and hypertension (Bernal-Mizrachi et al., 2007).

In totality, these observations highlight the importance of the vagal afferent pathway not only in short-term nutrient status alterations, such as blood glucose concentrations, but also in long-term energy storage status alterations.

Therapeutic possibilities. A recent study found a low resting metabolic rate to predict susceptibility to obesity (Buscemi et al., 2005). Therefore, enhancing energy expenditure is a promising strategy for treating obesity. Physiological sympathetic activation might thus be feasible, because it leads to relatively selective loss of fat, followed by improvements in insulin sensitivity beyond what would be expected from body weight reduction.

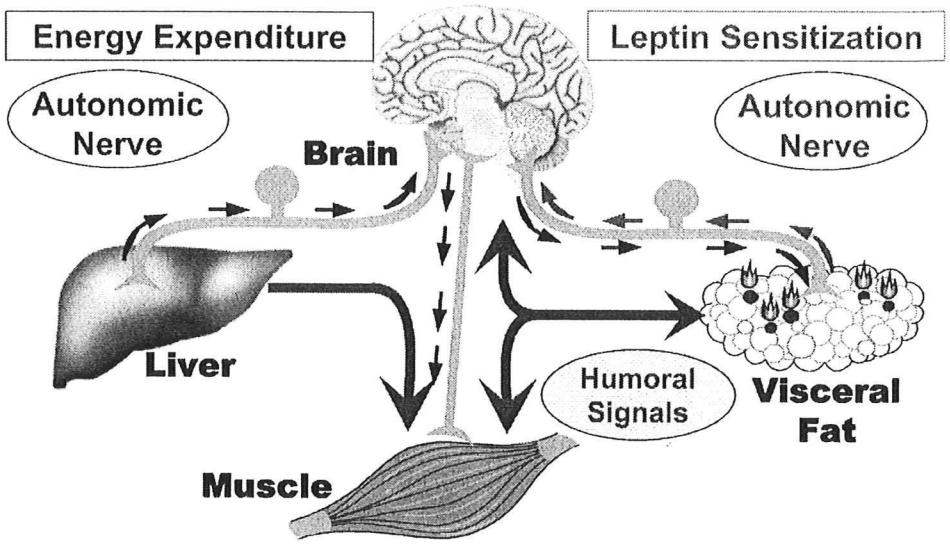

Fig. 2. Scheme of the neuronal pathways involved in energy homeostasis. Neuronal signals from WAT modulate hypothalamic leptin sensitivity, thereby regulating feeding behavior. In addition, the liver transmits information regarding excess energy to the brain via the afferent vagus, thereby activating the sympathetic nervous system which in turn enhances energy expenditure and lipolysis. The autonomic nervous system, as exemplified by these neuronal circuits, plays important roles in regulating energy metabolism.

Sibutramine, a serotonin-norepinephrine reuptake inhibitor, which is used clinically as an antiobesity drug, apparently increases sympathetic activity, though whether this would contribute in any way to weight loss in humans is unclear (Luque & Rey, 2002). As noted above, the autonomic nerve circuit consisting of the afferent vagus nerve and efferent sympathetic nervous system has a physiological antiobesity function exerted through enhanced energy expenditure. Therefore, in addition to reagents acting directly on the brain, activators of this peripheral nervous system are potential antiobesity drug targets. Elucidation of the underlying molecular mechanisms, including mediators influencing vagal activity, could lead to new therapeutic approaches to obesity and the metabolic syndrome. However, sibutramine raises both heart rate and blood pressure. Caution is necessary when any novel agents raises energy expenditure, because these effects may simply be due to an overall increase in systemic sympathetic activity.

The sympathetic nervous system reportedly activates uncoupling protein-1 (UCP-1) expression and activity. UCP-1 is most abundantly expressed in brown adipose tissue and dissipates energy as heat. In addition, transgenic overexpression of UCP-1 in WAT has been reported to exert preventive effects against the development of both genetic and dietary obesity, as well as the associated insulin resistance, in mice (Kopecky et al., 1995, 1996). Furthermore, hepatic induction of UCP-1 protein in mice with dietary obesity improves both diabetes and obesity by exerting local effects in the liver as well as remote effects in adipose tissues, muscle and the hypothalamus (Ishigaki et al., 2005). In contrast, in lean mice fed a standard diet, hepatic UCP-1 expression had little impact on either glucose or lipid metabolism and no cachectic phenotypes were observed (Ishigaki et al., 2005). These observations suggest UCP-1 to possibly be an attractive therapeutic target for both obesity and the metabolic syndrome.

2.2.2.3. Signals from adipose tissues. Few reports have focused on afferent nerve signals from adipose tissues. Niijima (1998) and Tanida et al. (2000) used electrical firing measurements to demonstrate that leptin induces functional activation of afferent nerve fibers from epididymal WAT. The functional roles of these afferent nerves in food intake regulation has been recently shown (Yamada et al., 2006).

Fat accumulation in intra-abdominal adipose tissue plays a major role in development of the metabolic syndrome, which is associated with insulin as well as leptin resistance. As described above, leptin resistance is induced by excessive adiposity and, in turn, is an important mechanism for maintaining the obese state. UCP-1 induction in restricted portions of epididymal adipose tissue, even at very low levels, dramatically improves hypothalamic leptin resistance without altering adiposity and thereby decreases food intake. Locally dissecting nerves from the epididymal fat pad and pharmacological deafferentation blunted these anorectic effects of UCP-1 expression in adipose tissue. Thus, afferent nerve signals originating in epididymal fat pads were shown to modulate hypothalamic sensitivity to leptin (Yamada et al., 2006; Fig. 2). In addition, the involvement of afferent nerves from WAT in adiposity was suggested by the observation that localized selective sensory denervation, achieved by microinjecting capsaicin bilaterally into epididymal WAT of Siberian hamsters, produced increases in other intraabdominal fat masses (Shi & Bartness, 2005).

Adipose tissues were long regarded as simply being passive fuel storage sites. However, the discovery of various adipocytokines, with leptin being the most important example, has raised adipose tissue to the status of a versatile endocrine gland. In addition, these aforementioned studies provide further evidence that adipose tissue serves as a base, sending out neuronal signals regulating feeding and energy storage.

Therapeutic possibilities. From the therapeutic perspective, the mechanism underlying leptin resistance is an important issue awaiting clarification, though two hypotheses have received considerable attention. One involves a failure of circulating leptin to arrive at its targets in the brain. Leptin is normally transported across the blood brain barrier by a saturable transport system, and the activity of this system has been shown to be impaired in obese subjects (Schwartz et al., 1996). Intranasal delivery of leptin can reportedly overcome this barrier and thereby produce weight loss in rats (Fliedner et al., 2006).

Another important observation is that, independently of blood–brain transit, intracellular leptin-receptor signaling is blunted in brain areas critical to energy homeostasis in the setting of diet-induced obesity, such that neuronal leptin responsiveness is diminished even when leptin is directly injected into the brain (El-Haschimi et al., 2000). In fact, several studies support potential roles of two molecules, suppressor of cytokine signaling-3 (SOCS3; Bjorbaek et al., 1998) and protein tyrosine phosphatase-1B (PTP1B; Cheng et al., 2002; Zabolotny et al., 2002), in the inhibitory regulation of Ob-Rb signaling both in vitro and in vivo. Although hypothalamic PTP1B levels do not appear to be altered in obesity, SOCS3 expression is increased in several rodent models of leptin-resistant obesity, which is consistent with the potential role of SOCS3 in leptin resistance (Bjorbaek et al., 1998; Munzberg et al., 2004). Moreover, ablation of SOCS3 activity by employing neuron-specific conditional knockout increases leptin-induced activation of intracellular signaling events and catabolic neuropeptide expressions, associated with enhancement of the weight-reducing effects of leptin and resistance to diet-induced obesity (Howard et al., 2004; Mori et al., 2004). PTP1B also inactivates the leptin receptor via dephosphorylation of its key tyrosine residues, which are phosphorylated in response to ligand binding (Cheng et al., 2002; Zabolotny et al., 2002). Global and neuron-specific PTP1B knockout mice are lean, resistant to diet-induced obesity, and insulin sensitive, all of which result more from increased energy expenditure than decreased food intake (Elchebly et al., 1999; Klaman et al., 2000; Bence et al., 2006). Therefore, SOCS3 and PTP1B are potential therapeutic targets for leptin resistance. Caution is warranted, however, since SOCS3 and PTP1B regulate more than just leptin signaling.

As stated above, neuronal signals from intraabdominal adipose tissue modulate hypothalamic leptin sensitivity (Yamada et al., 2006). Activation of this novel neuronal pathway is a possible therapeutic strategy against obesity and the metabolic syndrome. Elucidating the molecular mechanism(s) underlying this pathway, including identification of the neurotransmitters involved and their receptors, might lead to the development of novel therapeutic strategies, tackling the metabolic syndrome via improved leptin resistance.

3. Epilogue

Metabolism is not a process carried on independently in different organs/tissues, but rather is coordinated and regulated throughout the body. The coordination of metabolic regulation among organs/tissues, which requires communication among these organs/tissues, is apparently essential for maintaining the homeostasis of systemic metabolism, especially glucose and energy metabolism. In addition, disturbances of this coordinated control system may be involved in the development of metabolic disorders, including obesity, type 2 diabetes, hyperlipidemia, and the metabolic syndrome.

Recent research advances have revealed the complex and important roles played by the central nervous system. The brain obtains an abundance of metabolic information from peripheral organs/tissues through humoral and neuronal avenues. In addition, these signals interact, as exemplified by adiponectin expressions being regulated by sympathetic activity (Imai et al., 2006). These inputs are most likely integrated and processed in the brain, leading to the transmission of regulatory signals, which then induce appropriate systemic metabolic responses (Katagiri et al., 2007). Elucidation of these regulatory systems, in far greater detail, may reveal the mechanisms underlying metabolic homeostasis and thereby allow us to understand the complex metabolic disorders that result from perturbation of these systems.

Although life-style change is widely accepted as the first-line treatment for obesity and the metabolic syndrome, in the actual clinical setting, the multiple risks associated with obesity do not normalize with efforts aimed at life-style changes alone. Unfortunately, the existing pharmacological treatments for obesity, which might be used to ameliorate the risks associated with obesity, provide limited efficacy. This lack of efficacy is often further compounded by unacceptable side-effects. Concern about the safety of centrally acting drugs is one reason that pharmaceutical companies are currently seeking alternative obesity treatments. Targeting inter-tissue/organ communication in energy homeostasis might offer advantages in exploiting natural regulatory circuits while minimizing unwanted side effects. Diet and exercise remain the undisputed cornerstones of obesity therapy. However, more effective medications, designed to augment the impacts of these efforts, would be welcomed by obese individuals.

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Cell Metabolism
Short Article

ATF4-Mediated Induction of 4E-BP1 Contributes to Pancreatic β Cell Survival under Endoplasmic Reticulum Stress

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SUMMARY

Endoplasmic reticulum (ER) stress-mediated apoptosis may play a crucial role in loss of pancreatic β cell mass, contributing to the development of diabetes. Here we show that induction of 4E-BP1, the suppressor of the mRNA 5′ cap-binding protein eukaryotic initiation factor 4E (eIF4E), is involved in β cell survival under ER stress. 4E-BP1 expression was increased in islets under ER stress in several mouse models of diabetes. The *Eif4ebp1* gene encoding 4E-BP1 was revealed to be a direct target of the transcription factor ATF4. Deletion of the *Eif4ebp1* gene increased susceptibility to ER stress-mediated apoptosis in MIN6 β cells and mouse islets, which was accompanied by deregulated translational control. Furthermore, *Eif4ebp1* deletion accelerated β cell loss and exacerbated hyperglycemia in mouse models of diabetes. Thus, 4E-BP1 induction contributes to the maintenance of β cell homeostasis during ER stress and is a potential therapeutic target for diabetes.

INTRODUCTION

Recent studies have shown decreased pancreatic β cell mass to be a common feature of subjects with type 2 diabetes mellitus (Butler et al., 2003). Susceptibility to stress-induced apoptosis may underlie β cell loss. Translational regulation is an essential strategy by which cells cope with stress conditions (Clemens, 2001). Translation of eukaryotic mRNA is regulated primarily at the level of initiation. Translational initiation begins with formation of a ternary complex composed of the methionine-charged initiator tRNA, eukaryotic initiation factor 2 (eIF2), and GTP (Holcik and Sonenberg, 2005). The ternary complex then binds to the 40S ribosomal subunit and several other initiation factors, generating the 43S preinitiation complex. The mRNA 5′ cap-binding protein eIF4E associates with eIF4A and eIF4G to form the eIF4F complex and interacts with the 5′ cap structure of the mRNA. The eIF4F complex then recruits the 43S preinitiation complex to the mRNA, allowing the complex to scan toward the initiator AUG codon. The two best characterized regulatory steps in this translational control are formation of the ternary complex and assembly of the eIF4F complex. Phosphorylation of the α subunit of eIF2 (eIF2α) prevents ternary complex formation and thereby suppresses global translation. In addition, eIF4E-binding proteins (4E-BPs) inhibit eIF4F assembly by competitively displacing eIF4G from eIF4E. Global translational suppression through eIF2α phosphorylation is a mechanism shared among different stress-response pathways. Depending on the nature of the stress stimulus, eIF2α can be phosphorylated by four different kinases (Holcik and Sonenberg, 2005). Global attenuation of protein biosynthesis then paradoxically increases expression of several proteins, including the transcription factor ATF4 (Harding et al., 2000).

Because of their high insulin secretory activity, β cells are vulnerable to endoplasmic reticulum (ER) stress, a condition of disrupted ER homeostasis due to accumulation of misfolded proteins (Schroder and Kaufman, 2005). Cells respond to ER stress by activating an adaptive cellular response known as the unfolded protein response (UPR). Under ER stress conditions, global translation is suppressed through eIF2α phosphorylation by an ER-resident kinase, PERK. The importance of PERK-mediated translational suppression has been demonstrated in infancy-onset diabetes and skeletal defects caused by loss of PERK in humans (Delepine et al., 2000) and mice (Harding et al., 2001; Zhang et al., 2002). However, the roles of translational control through inhibition of eIF4F assembly by 4E-BPs under stress conditions, including ER stress, have yet to be fully clarified. Herein, we have studied roles of 4E-BP1,

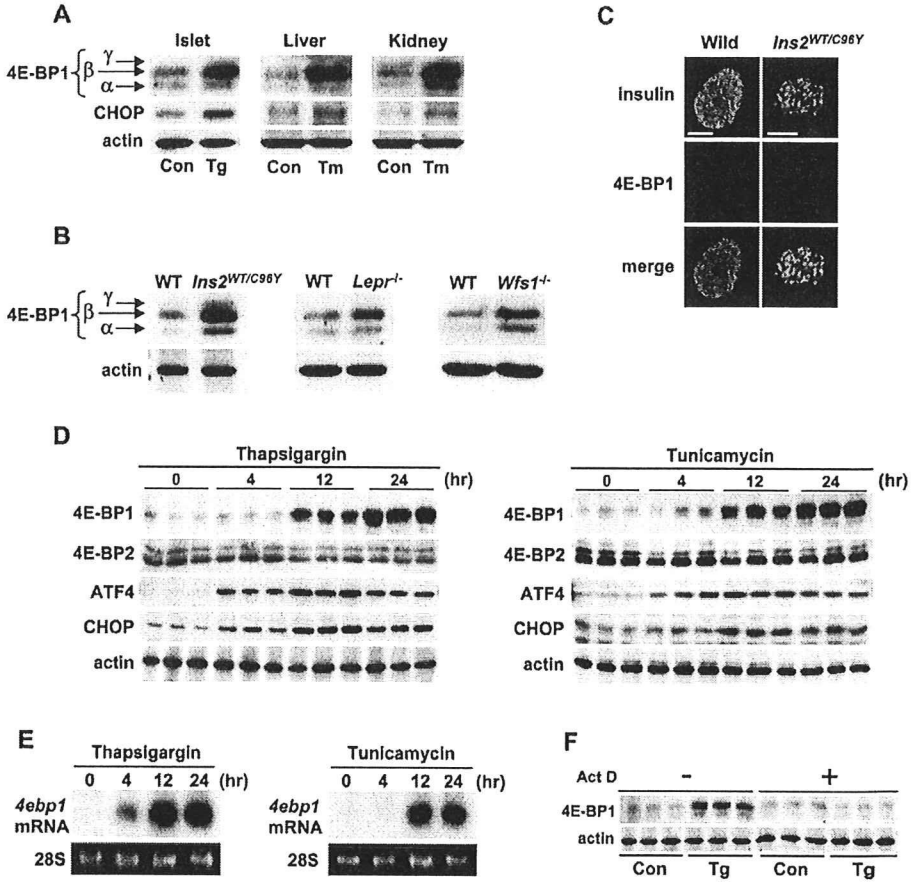

Figure 1. ER Stress Induces 4E-BP1 Expression

(A) Expression of 4E-BP1 protein in isolated islets treated with vehicle (0.05% DMSO) control (Con) or 0.5 μM thapsigargin (Tg) for 12 hr. 4E-BP1 expression was also examined in the livers and kidneys of mice that had received intraperitoneal injections of tunicamycin (Tm) 96 hr previously.

(B) Expression of 4E-BP1 protein in islets from wild-type (WT), *Ins2*$^{WT/C96Y}$, *Lepr*$^{-/-}$, and *Wfs1*$^{-/-}$ mice.

(C) Immunostaining of pancreatic sections from WT and *Ins2*$^{WT/C96Y}$ mice using anti-insulin and anti-4E-BP1 antibodies. Scale bars = 50 μm.

(D and E) Time courses of 4E-BP1, 4E-BP2, ATF4, and CHOP expression (D) and *4ebp1* mRNA expression (E) in MIN6 cells treated with thapsigargin (left panel) or tunicamycin (right).

(F) Inhibition of 4E-BP1 induction by actinomycin D (1 μg/ml) in MIN6 cells treated with thapsigargin for 12 hr.

one of three isoforms of the 4E-BP family, in β cells under ER stress.

RESULTS

ER Stress Induces 4E-BP1

4E-BP1 protein is present in three forms with different phosphorylation states. The hypophosphorylated α and β forms are active and the hyperphosphorylated γ form is inactive in terms of eIF4E binding. Expression of 4E-BP1 protein, especially the hypophosphorylated forms, was markedly induced, with an increase in CHOP, a stress marker protein, in isolated islets treated with thapsigargin (an ER Ca^{2+} pump inhibitor causing ER stress) (Figure 1A). 4E-BP1 induction was also observed in liver and kidneys of mice administered tunicamycin (a protein glycosylation inhibitor), another ER stress inducer (Figure 1A).

Furthermore, 4E-BP1 protein expression was markedly increased in *Ins2*$^{WT/C96Y}$ islets (Figures 1B and 1C), in which misfolded insulin molecules with a C96Y mutation cause ER stress (Wang et al., 1999). Islets from leptin receptor null (*Lepr*$^{-/-}$) mice, which have been shown to suffer from ER stress (Laybutt et al., 2007), also exhibited increased 4E-BP1 expression (Figure 1B). The *Wfs1*$^{-/-}$ mouse (Ishihara et al., 2004) is a model of Wolfram syndrome, which is characterized by juvenile-onset diabetes mellitus and optic atrophy and is caused by *WFS1* mutations (Inoue et al., 1998; Strom et al., 1998). WFS1-deficient islets are affected by chronic ER stress (Ishihara et al., 2004; Riggs et al., 2005). Again, 4E-BP1 protein was increased in *Wfs1*$^{-/-}$ islets (Figure 1B).

Induction of 4E-BP1 by ER stress was also observed in insulinoma MIN6 cells (Miyazaki et al., 1990) (Figure 1D). Expression of 4E-BP2, another member of the 4E-BP family, remained unchanged. While expression of ATF4 and CHOP peaked at 12 hr after treatment with thapsigargin or tunicamycin, 4E-BP1 protein was further increased at 24 hr posttreatment (Figure 1D). 4E-BP1 protein induction appeared to result from transcriptional

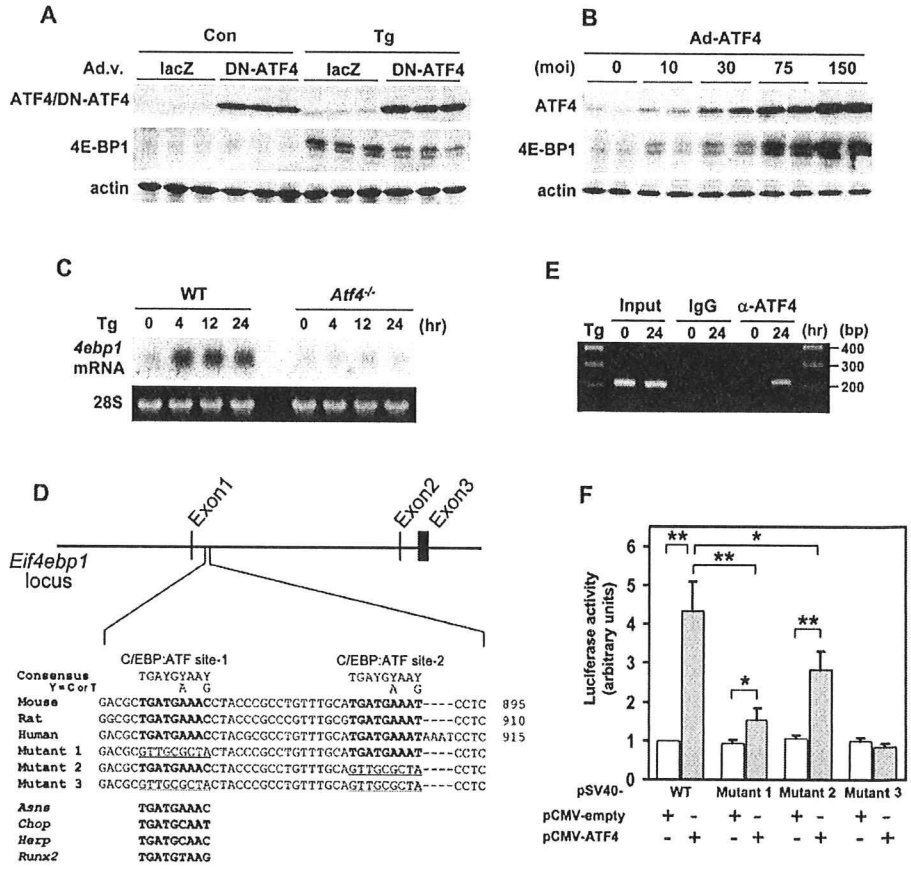

Figure 2. *Eif4ebp1* Is a Direct Target of ATF4

(A) Suppression of thapsigargin (Tg, 0.5 μM)-induced 4E-BP1 expression by dominant-negative ATF4 (DN-ATF4). MIN6 cells were infected with an adenovirus expressing either lacZ or DN-ATF4. Two days later, the cells were treated with vehicle (0.05% DMSO) control (Con) or Tg for 12 hr.

(B) 4E-BP1 expression in MIN6 cells infected with an adenovirus expressing wild-type ATF4 at the indicated multiplicity of infection (moi).

(C) *4ebp1* mRNA levels in wild-type and *Atf4*$^{-/-}$ MEFs treated with thapsigargin.

(D) C/EBP:ATF composite sites in intron 1 of the *Eif4ebp1* gene. Mouse, rat, and human *Eif4ebp1* gene segments are aligned with ATF4 binding sequences in several genes. Numbers are positions relative to A of the initial ATG codon. *Asns*, asparagine synthetase; *Herp*, homocysteine-induced ER protein; *Runx2*, runt-related transcription factor 2.

(E) Chromatin immunoprecipitation assay of MIN6 cells treated with thapsigargin. DNAs precipitated with nonspecific or anti-ATF4 IgG were amplified using primers for the *Eif4ebp1* intron 1 region.

(F) ATF4 induction of luciferase reporters with the *SV40* promoter and an *Eif4ebp1* gene segment with C/EBP:ATF composite sites or their mutants shown in (D). MIN6 cells were transfected with luciferase reporters together with either pCMV-empty or pCMV-ATF4. Error bars represent SEM. n = 4; *p < 0.05, **p < 0.01.

activation since *4ebp1* mRNA levels were also increased by these ER stress inducers (Figure 1E) and the transcriptional inhibitor actinomycin D completely blocked 4E-BP1 induction by thapsigargin (Figure 1F).

ATF4 Directly Activates the *Eif4ebp1* Gene

MIN6 cells were infected with recombinant adenoviruses expressing dominant-negative (DN) forms of transcription factors involved in the UPR. Expression of DN-ATF4 (He et al., 2001) (Figure 2A), but not DN-ATF6 or DN-XBP1 (see Figure S1 available online), suppressed 4E-BP1 induction by thapsigargin. Conversely, expression of wild-type ATF4 dramatically induced 4E-BP1 expression (Figure 2B). Furthermore, *4ebp1* mRNA levels were not increased by thapsigargin in *Atf4*$^{-/-}$ murine embryonic fibroblasts (MEFs) (Harding et al., 2003) (Figure 2C).

A survey of the mouse *Eif4ebp1* gene using a luciferase assay identified a segment in intron 1 that conferred thapsigargin sensitivity to a luciferase reporter (Figure S2). Indeed, we found two potential ATF4 binding sequences (C/EBP:ATF composite sites) in this segment (Figure 2D). Chromatin immunoprecipitation (ChIP) assays revealed that ATF4 binds this segment (Figure 2E). Furthermore, cotransfection of a luciferase reporter containing the C/EBP:ATF sites with an ATF4-expressing plasmid increased luciferase activity by 4.3-fold (Figure 2F). Disruption of the upstream C/EBP:ATF site (mutant 1) or the downstream site (mutant 2) decreased the ATF4-mediated increase in luciferase activity by 83% or 47%, respectively, and disruption of both (mutant 3) completely abolished the increase (Figure 2F).

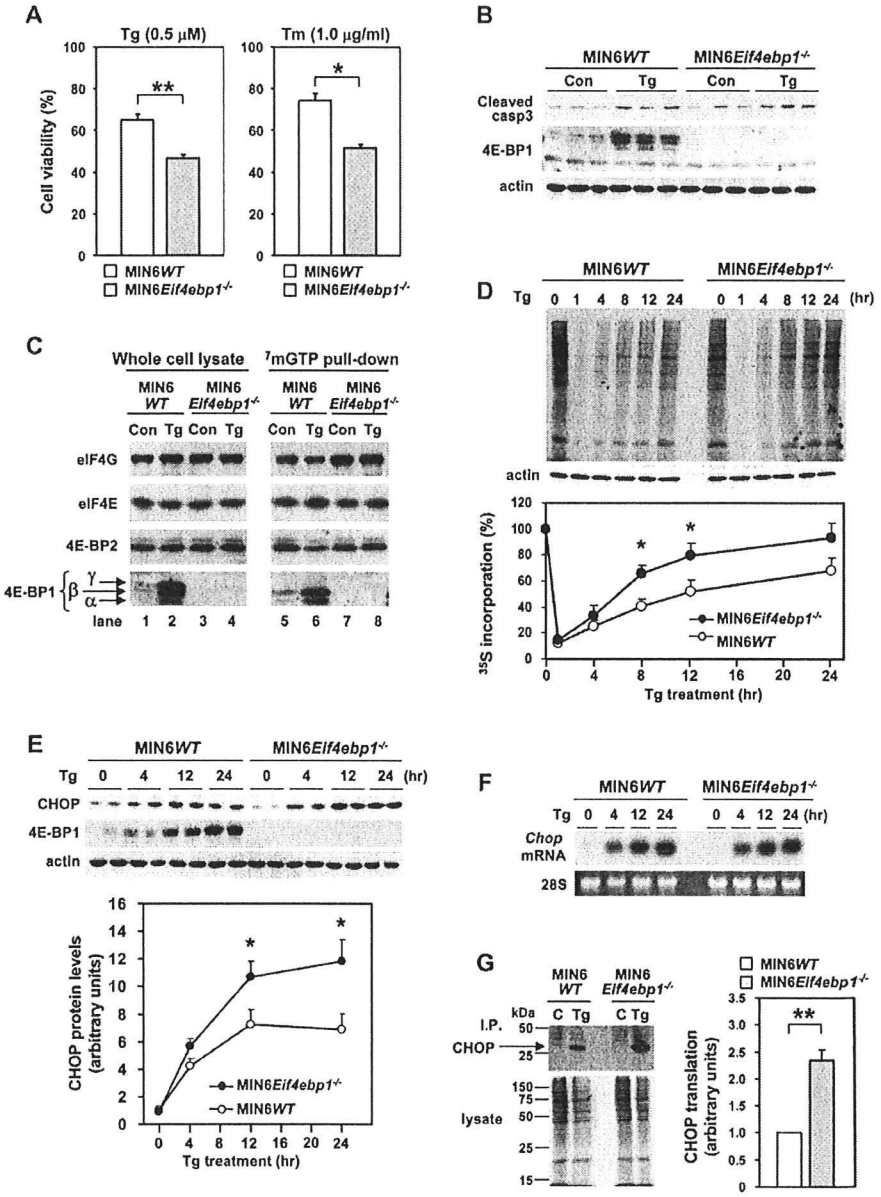

Figure 3. 4E-BP1-Deficient Cells Exhibit Increased Apoptosis Susceptibility with Deregulated Translational Control

(A) Viability of MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with 0.5 μM thapsigargin (Tg) or 1.0 μg/ml tunicamycin (Tm) for 36 hr, normalized to MIN6*WT* cells treated with vehicle (0.05% DMSO). n = 3–4.

(B) Immunoblot of cleaved caspase-3 in MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with vehicle control (Con) or thapsigargin for 24 hr.

(C) Immunoblot analysis of 4E-BP1, 4E-BP2, eIF4E, and eIF4G in whole-cell lysates (left) or in a complex associated with ^{7}mGTP-Sepharose (right) in cells treated with thapsigargin for 24 hr.

(D) [^{35}S]methionine/cysteine incorporation during a 15 min pulse labeling in MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells pretreated with thapsigargin for the indicated periods. Ten percent of the lysates were also probed with an anti-actin antibody. A representative autoradiogram is shown in the upper panel; data from three experiments are summarized in the lower panel.

(E) Increased CHOP induction in MIN6*Eif4ebp1*$^{-/-}$ cells treated with thapsigargin. Representative blots are shown in the upper panel; data from four experiments are summarized in the lower panel.

(F) *Chop* mRNA levels in MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with thapsigargin.

(G) Greater *Chop* translation in MIN6*Eif4ebp1*$^{-/-}$ cells treated with thapsigargin. MIN6*WT* and MIN6*Eif4ebp1*$^{-/-}$ cells treated with vehicle (C) or thapsigargin (Tg) for 12 hr were labeled with [^{35}S]methionine/cysteine. Lysates were either directly subjected to SDS-PAGE or immunoprecipitated with anti-CHOP antibody. Representative autoradiograms are shown in the left panel; data from four experiments are summarized in the right panel.

Error bars represent SEM. *$p < 0.05$, **$p < 0.01$.

4E-BP1-Deficient β Cells Are More Vulnerable to ER Stress

A 4E-BP1-deficient β cell line, MIN6*Eif4ebp1*$^{-/-}$, was established by crossing *Eif4ebp1*$^{-/-}$ mice (Tsukiyama-Kohara et al., 2001) with IT6 mice expressing SV40 large T antigen in β cells (Miyazaki et al., 1990). MIN6 cells with wild-type *Eif4ebp1* alleles, established in parallel, were designated MIN6*WT* cells. MIN6*Eif4ebp1*$^{-/-}$ cells were more vulnerable to ER stress inducers than MIN6*WT* cells (Figure 3A). 4E-BP1 re-expression restored this diminished viability of MIN6*Eif4ebp1*$^{-/-}$ cells to control levels (Figure S3A). The increased susceptibility to ER stress-induced cell death was accompanied by enhanced caspase-3 cleavage (Figure 3B), indicating that the reduced viability of MIN6*Eif4ebp1*$^{-/-}$ cells was due at least in part to increased apoptosis. In addition, DNA fragmentation under ER stress was greater in *Eif4ebp1*$^{-/-}$ islets than in wild-type islets (Figure S3B). These results suggest that 4E-BP1 induction contributes to β cell survival under ER stress.

We then examined the impact of 4E-BP1 deficiency on the integrity of the eIF4F translational initiation complex. Pull-down assays of eIF4E and its binding partners with a cap analog, 7-methyl-GTP, revealed that thapsigargin-induced 4E-BP1 expression resulted in marked increases in the amounts of hypophosphorylated 4E-BP1 α and β forms bound to eIF4E, displacing eIF4G from eIF4E in MIN6*WT* cells (Figure 3C, compare lane 5 with lane 6). The amount of eIF4G bound to eIF4E was reduced to 63% ± 3% (n = 4, $p < 0.05$) of that in vehicle-treated MIN6WT cells. In contrast, levels of eIF4G bound to eIF4E were not decreased by thapsigargin in MIN6*Eif4ebp1*$^{-/-}$ cells (Figure 3C, compare lane 7 with lane 8). Thus, eIF4E availability for translational initiation was greater in MIN6*Eif4ebp1*$^{-/-}$ cells than in MIN6*WT* cells under ER stress. Measurement of the global translation rate revealed that recovery from translational suppression by thapsigargin was more rapid in 4E-BP1-deficient cells (Figure 3D).

Translation of newly synthesized mRNA molecules is reportedly much more dependent on eIF4E availability than that of preexisting mRNAs (Novoa and Carrasco, 1999). Expression of CHOP, a mediator of ER stress-induced apoptosis, was thus studied in MIN6*Eif4ebp1*$^{-/-}$ cells since *Chop* mRNA is one of the transcripts most abundantly synthesized during ER stress (Pirot et al., 2007). *Eif4ebp1* deletion caused greater CHOP protein induction by thapsigargin in MIN6 cells (Figure 3E), with unaltered *Chop* mRNA accumulation (Figure 3F). Pulse-labeling experiments demonstrated enhanced CHOP translation (Figure 3G). Thus, CHOP expression during ER stress was augmented via increased translation in 4E-BP1 deficiency.

Eif4ebp1 Deletion Accelerates β Cell Loss in Mouse Diabetes Models

To examine the roles of 4E-BP1 under ER stress in vivo, *Eif4ebp1*$^{-/-}$ mice on the 129S6 background were fed a high-fat diet (HFD), which is thought to produce ER stress in β cells through peripheral insulin resistance (Scheuner et al., 2005). *Eif4ebp1*$^{-/-}$ mice developed glucose intolerance (Figures S4A and S4B), which was associated with blunted insulin secretion (Figure S4C) and reduced pancreatic insulin content (Figure S4D) as compared to HFD-fed wild-type mice. These data suggest that *Eif4ebp1*$^{-/-}$ mice have a β cell defect. However, HFD-fed *Eif4ebp1*$^{-/-}$ mice gained more weight and were more insulin resistant than HFD-fed wild-type mice (Figures S4E and S4F). Therefore, the possibility remains that β cell failure in HFD-fed *Eif4ebp1*$^{-/-}$ mice resulted from greater ER stress rather than from a defect in β cells lacking 4E-BP1.

We next crossed *Eif4ebp1*$^{-/-}$ mice with two genetic models of diabetes in which β cells are under ER stress, *Ins2*$^{WT/C96Y}$ and *Wfs1*$^{-/-}$ mice on the 129S6 background. 4E-BP1 deficiency did not alter body weight (Figures S5A and S5B) or insulin sensitivity (Figures S5C and S5D) but worsened hyperglycemia in *Ins2*$^{WT/C96Y}$ (Figure 4A) and *Wfs1*$^{-/-}$ (Figure 4B) mice. In *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ mice, pancreatic insulin content was less than half of that in *Ins2*$^{WT/C96Y}$ mice at 5 weeks of age (Figure 4C), and the majority of islets in *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ mice were smaller as compared to those in *Ins2*$^{WT/C96Y}$ mice (Figure 4D). We also observed a 38% decrease in pancreatic insulin content in *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ mice as compared to *Wfs1*$^{-/-}$ mice (Figure 4E). Importantly, the insulin-positive area was smaller in pancreatic sections from *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ mice than in pancreatic sections from *Wfs1*$^{-/-}$ mice at 27–30 weeks of age (Figure 4F), indicating that ER stress-mediated β cell loss is exacerbated by 4E-BP1 deficiency in vivo.

Global protein synthesis was studied in these mouse islets. A tendency toward decreased protein synthesis was observed in both *Ins2*$^{WT/C96Y}$ (Figure 4G, hatched bar; $p = 0.074$) and *Wfs1*$^{-/-}$ islets (Figure 4H, hatched bar; $p = 0.079$) as compared to wild-type islets. *Eif4ebp1* deletion ablated this regulation and resulted in significantly increased protein synthesis in *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ ($p = 0.013$) and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ ($p = 0.045$) islets as compared to that in corresponding single mutants (compared hatched with filled bars in Figures 4G and 4H). These data suggest that accelerated β cell loss under ER stress is due to deregulated translational control.

DISCUSSION

Our results implicate 4E-BP1, identified as a component of the UPR, in β cell survival under ER stress. Important roles of 4E-BPs under various stress conditions have been recently demonstrated in yeast (Ibrahimo et al., 2006) and *Drosophila* (Teleman et al., 2005; Tettweiler et al., 2005). These data suggest that translational suppression by 4E-BPs is an evolutionarily conserved strategy against stress conditions. Although we focused on β cells, ER stress-mediated induction of 4E-BP1 was also observed in the liver and kidneys, suggesting the general importance of the present findings.

Our results suggest that, in addition to translational regulation by eIF2α phosphorylation due to PERK activation, another mode of translational control mediated by 4E-BP1 plays a role in the maintenance of β cell homeostasis under ER stress. Since translational suppression by eIF2α phosphorylation is transient owing to feedback dephosphorylation by GADD34 (Novoa et al., 2001), prolonged translational suppression by 4E-BP1 might be needed in the later stages of the UPR. However, in contrast to PERK, 4E-BP1 deficiency alone does not cause diabetes in mice under normal conditions, suggesting that 4E-BP1 protein is not a key regulator but rather functions with other molecules to maintain β cell homeostasis under ER stress. The preferential role of 4E-BP1 in the later stages of the UPR might be puzzling since expression of

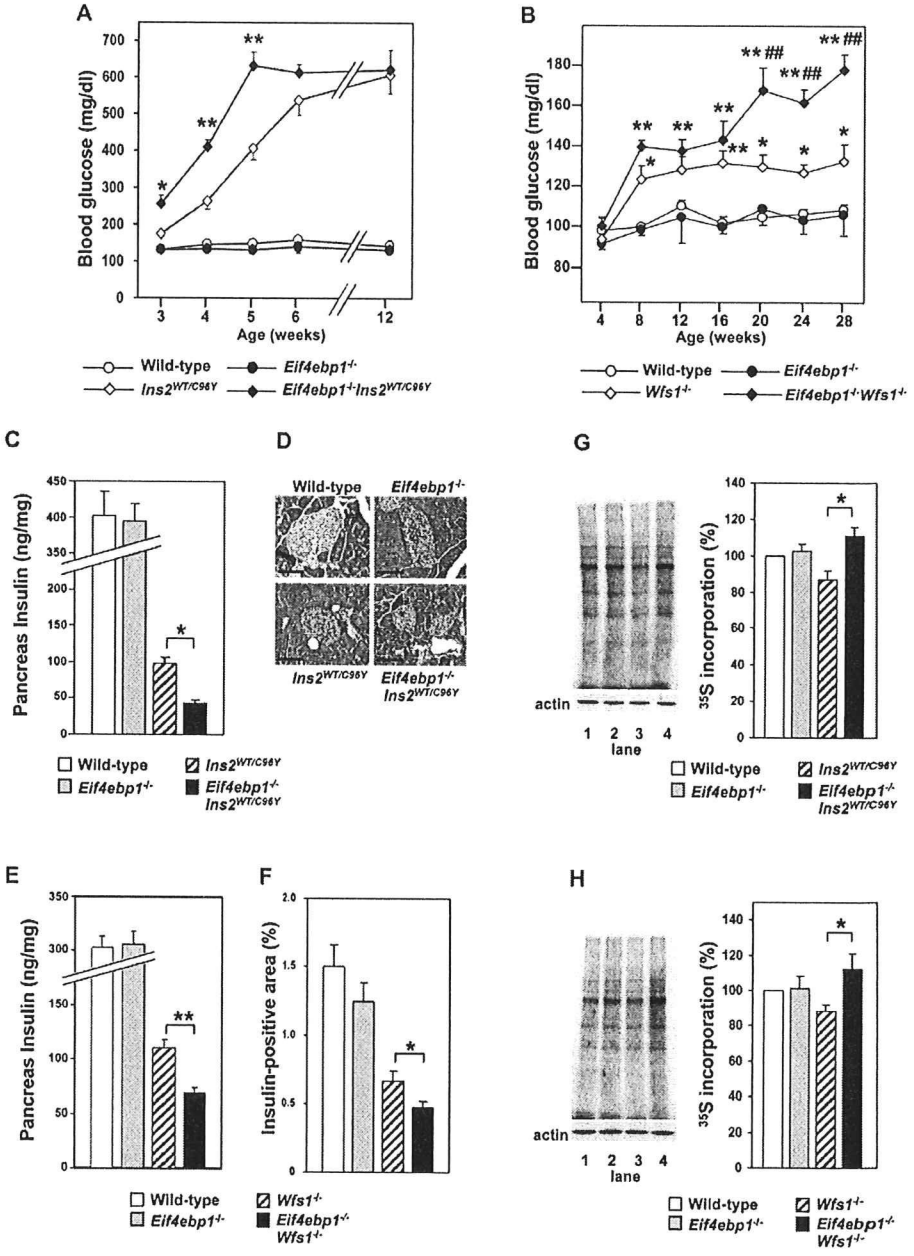

Figure 4. β Cell Loss Is Exacerbated by 4E-BP1 Deficiency in Mouse Diabetes Models

(A) Fed blood glucose levels of wild-type (n = 6), *Eif4ebp1*$^{-/-}$ (n = 5), *Ins2*$^{WT/C96Y}$ (n = 9), and *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$ (n = 11) mice. Data from three cohorts are combined. *p < 0.05, **p < 0.01 versus *Ins2*$^{WT/C96Y}$ mice.

(B) Fed blood glucose levels of wild-type (n = 12), *Eif4ebp1*$^{-/-}$ (n = 8), *Wfs1*$^{-/-}$ (n = 15), and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ (n = 10) mice. Data from three cohorts are combined. *p < 0.05, **p < 0.01 versus wild-type mice; ##p < 0.01 versus *Wfs1*$^{-/-}$ mice.

(C) Pancreatic insulin content of mice of the indicated genotypes at 5 weeks of age. n = 3 for each genotype. *p < 0.05.

(D) Hematoxylin and eosin staining of sections showing representative islets from mice of the indicated genotypes at 5 weeks of age. Scale bars = 50 μm.

(E) Pancreatic insulin content of wild-type (n = 8), *Eif4ebp1*$^{-/-}$ (n = 4), *Wfs1*$^{-/-}$ (n = 15), and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ (n = 12) mice at 27–30 weeks of age. **p < 0.01.

(F) Insulin-positive area in pancreatic sections of wild-type (n = 3), *Eif4ebp1*$^{-/-}$ (n = 3), *Wfs1*$^{-/-}$ (n = 4), and *Eif4ebp1*$^{-/-}$ *Wfs1*$^{-/-}$ (n = 5) mice at 27–30 weeks of age. *p < 0.05.

(G) [^{35}S]methionine/cysteine incorporation in islets of the indicated genotypes at 5–6 weeks of age. Ten percent of the lysates were also probed with an anti-actin antibody. A representative autoradiogram is shown in the left panel. Lane 1, wild-type; lane 2, *Eif4ebp1*$^{-/-}$; lane 3, *Ins2*$^{WT/C96Y}$; lane 4, *Eif4ebp1*$^{-/-}$ *Ins2*$^{WT/C96Y}$. Data from four experiments are summarized in the right panel. *p < 0.05.