Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1

Review

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Nutrient overload leads to obesity, insulin resistance, and often type 2 diabetes. Whereas increased fat intake is commonly cited as the major factor in diet-induced dysmetabolic states, increased protein consumption also contributes, through elevated circulating amino acids. Recent studies have revealed that ribosomal protein S6 kinase 1, S6K1, an effector of mTOR, is sensitive to both insulin and nutrients, including amino acids. Although S6K1 is an effector of growth, recent reports show that amino acids also negatively affect insulin signaling through mTOR/S6K1 phosphorylation of IRS1. Moreover, rather than signaling through the class 1 PI3K pathway, amino acids appear to mediate mTOR activation through class 3 PI3K, or hVps34. Consistent with this, infusion of amino acids into humans leads to S6K1 activation, inhibition of insulin-induced class 1 PI3K activation, and insulin resistance. Thus, S6K1 may mediate deleterious effects, like insulin resistance, and potentially type 2 diabetes in the face of nutrient excess.

Introduction

It is hypothesized that during evolution the scarcity of food led to the development of dominant genetic mechanisms to secure and manage caloric intake, mechanisms which prevailed over those that suppressed food intake (Neel, 1999). In Western societies, the increase in food availability has exposed these dominant genetic traits, with obesity emerging as a prevalent metabolic disorder (Must et al., 1999). One important pathogenic consequence of obesity is insulin resistance, which plays a central role in the development of type 2 diabetes and the onset of cardiovascular disease, both major causes of morbidity and rising heath care costs in western societies (Morgan et al., 2004). Thus, nutrient overload, in conjunction with genetic predispositions, may play a key role in the development of insulin resistance. Furthermore, while increased fat intake is frequently considered to be the major cause of metabolic abnormalities due to overnutrition, there is increasing evidence that elevated dietary protein consumption also contributes to this syndrome (Krebs, 2005). This observation is consistent with the approximate 33% rise in the consumption of processed meat over the last 50 years and with the association of high-protein diets with glucose intolerance, insulin resistance, and an increased incidence of type 2 diabetes (Linn et al., 1996, 2000; Schulze et al., 2003; Song et al., 2004). Moreover, in insulin-resistant states of obesity, plasma concentrations of amino acids are elevated, particularly branched-chain amino acids (Felig et al., 1969, 1970, 1974). These findings are in agreement with studies demonstrating that the infusion of amino acids induces insulin resistance in experimental settings, similar to what is observed with lipid administration (Krebs et al., 2002, 2003). These findings raise the possibility of a broad role for dietary protein excess in metabolic disease. Therefore the molecular mechanisms by which excess amino acids lead to the development of insulin resistance have important implications for public health and clinical medicine. Here we review recent evidence demonstrating that ribosomal protein S6 kinase 1 (S6K1), a component of

the insulin signal transduction pathway, through negatively regulating IRS1 function (Zick, 2004), may be a key molecular element in driving insulin resistance under conditions of nutrient overload (Patti and Kahn, 2004; Harrington et al., 2004; Shah et al., 2004; Tremblay et al., 2005; Um et al., 2004). It has been speculated that such a system may have evolved to prevent cells from actively taking up nutrients in response to insulin under conditions of nutrient overload, such as in the obese setting (Um et al., 2004).

S6K1

Insulin-induced anabolic responses, most notably ribosome biogenesis and protein synthesis, are dependent on nutritional state (Hay and Sonenberg, 2004). In turn, these responses rely in part on S6K1, a member of the A, G, and C family of serine/threonine protein kinases (Hanks and Hunter, 1995; Manning et al., 2002). In mammals there are two isoforms of S6K1, which are produced from the same transcript by alternative initiation translation start sites (Grove et al., 1991; Reinhard et al., 1992). The larger isoform, termed S6K1L, contains an amino-terminal 23 amino acid extension (Grove et al., 1991; Reinhard et al., 1992), which localizes S6K1L to the nucleus. In contrast, the shorter form, S6K1S, appears to be largely localized to the cytoplasm (Reinhard et al., 1994). Findings to date would suggest that the two isoforms are controlled in a similar manner and are thus collectively referred to here as S6K1. Deletion of the S6K1 gene in mice led to the discovery of a second S6 kinase gene (Shima et al., 1998), highly identical to the S6K1 gene, termed S6K2 or S6Kβ (Shima et al., 1998; Gout et al., 1998; Saitoh et al., 1998). S6K2 shows 82% identity in the catalytic domain and in the linker and autoinhibitory domains with S6K1 (Gout et al., 1998; Saitoh et al., 1998; Shima et al., 1998). Moreover, there are apparently two isoforms, derived by alternate splicing, that we have termed here S6K2L, the larger, and S6K2S, the smaller (Gout et al., 1998; Lee-Fruman et al., 1999). Both S6K1 and 2 are widely expressed. S6K1 is identical
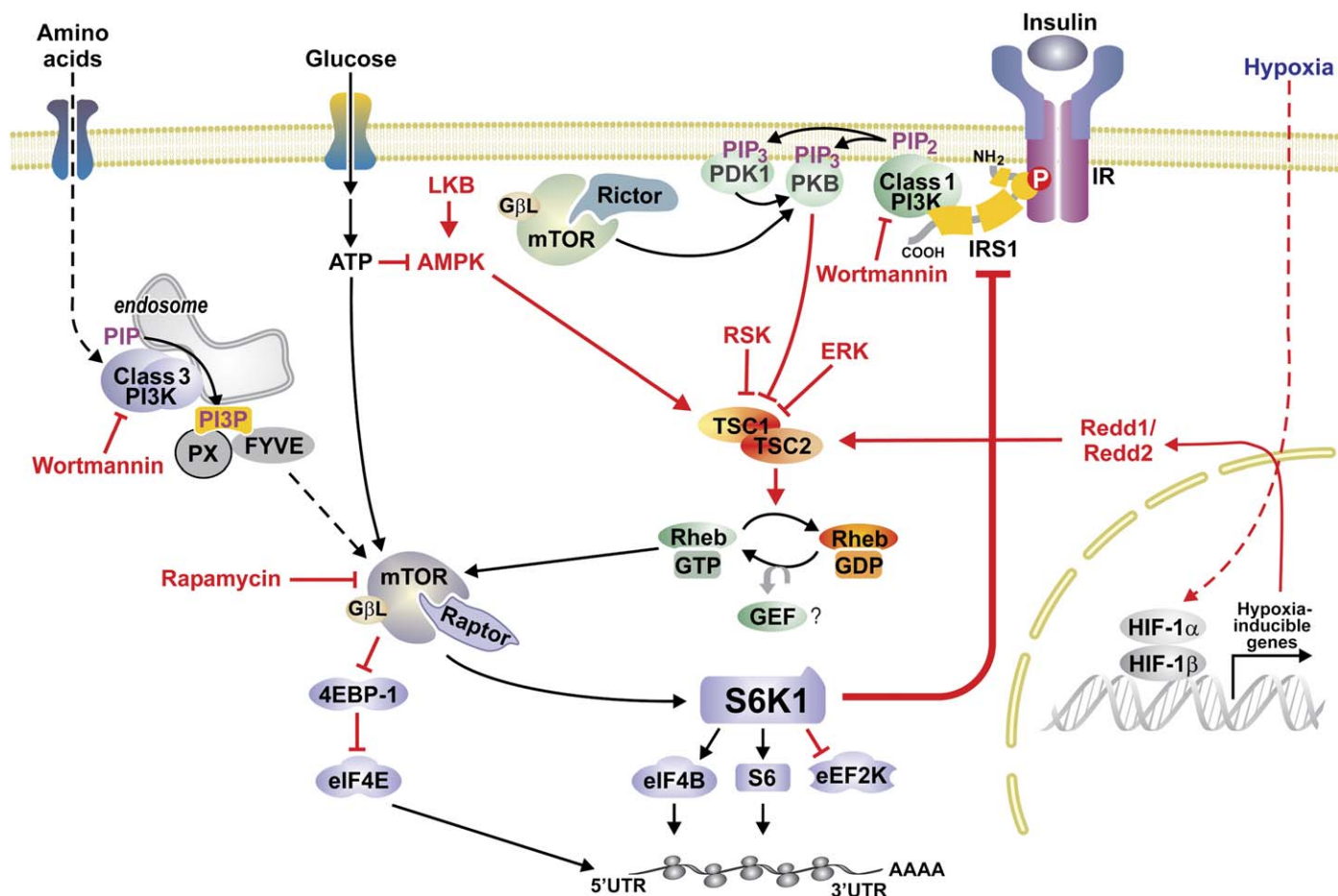
Figure 1. Model depicting mTOR/S6K1 signaling pathway in response to nutrients and insulin

The mTOR/S6K1 signaling pathway mediates various biological effects of nutrients, insulin, and energy. Nutrients such as amino acids and glucose increase hVps34 activity, stimulating the production of PI3P. PI3P recruits proteins containing FYVE or PX domains to endosomal membranes. The mTOR/S6K1 signaling pathway is also activated by insulin. Insulin binds the insulin receptor and leads to an increase in tyrosine phosphorylation of IRS1. IRS1-associated class 1 PI3K increases the production of PIP_3, recruiting PDK1 and PKB to the plasma membrane. PKB is activated by the concerted action of the rapamycin-insensitive rictor-mTOR complex and PDK1. Activated PKB phosphorylates and subsequently inactivates TSC2, a GTPase-activating protein, leading to an increase in GTP bound Rheb. Rheb-GTP increases mTOR activity and further facilitates the phosphorylation of S6K1 and 4E-BP1. The mTOR/S6K1 signaling pathway can sense energy levels either directly, through mTOR, or indirectly, through AMPK. Energy stress, such as fasting or lowering cellular ATP concentration, induces AMPK activation, leading to a PKB-independent TSC2 phosphorylation, which is thought to activate TSC2. This activation of TSC2 results in an increase in GDP bound Rheb and subsequent decrease in mTOR activity. Hypoxia induces Redd1/2, leading to a decrease of mTOR activity in a pathway parallel to the AMPK. Dashed lines indicate an interaction with an unknown mechanism. Red lines indicate inhibitory signal to the mTOR/S6K1 pathway.

in amino acid sequence in all mammalian species examined to date, whereas S6K2 displays a high degree of identity (Montagne and Thomas, 2004). The homology extends to invertebrates in which a single-copy ortholog was identified for the S6Ks in *Drosophila melanogaster* (Stewart et al., 1996; Watson et al., 1996). Researchers have also found single-copy orthologs in *Caenorhabditis elegans* (Long et al., 2002), *Aplysia californica* (Khan et al., 2001), *Artemia franciscana* (Santiago and Sturgill, 2001), and *Schizosaccharomyces pombe* (Matsuo et al., 2003). In addition, two S6K1 and S6K2 orthologs are present in *Arabidopsis thaliana* (Turck et al., 1998), indicating that S6 kinases are evolutionarily conserved in plants. Downstream a number of effectors have been identified, including 40S ribosomal protein S6 (Montagne and Thomas, 2004), protein synthesis initiation factor 4B (Raught et al., 2004), and elongation factor 2 kinase (Proud, 2004) (Figure 1).

Insulin signaling to S6K1

Insulin induces S6K1 activation through a canonical signal transduction pathway (Hay and Sonenberg, 2004; Montagne and Thomas, 2004), which is initiated by insulin receptor (IR) autophosphorylation and the recruitment and phosphorylation of IR substrates 1 and 2 (IRS1/2) at multiple tyrosine residues (Figure 1) (White, 1998). A subset of these sites serve as docking motifs for the p85 regulatory subunit of class 1, phosphoinositide 3-kinase (PI3K). This leads to the production of phosphatidylinositide-3, 4, 5-P_3 (PIP_3), which binds to the pleckstrin homology (PH) domain of protein kinase B (PKB), disrupting its interaction with the catalytic domain. The phosphoinositide-dependent protein kinase 1 (PDK1) can then phosphorylate PKB T308 and activate the kinase (Alessi et al., 1997; Stokoe et al., 1997) (Figure 1). PKB subsequently phosphorylates Tuberous Sclerosis Complex protein 2 (TSC2), inducing the

degradation of the tumor suppressor complex made up of TSC2 and TSC1 (Dan et al., 2002). In the active state, TSC1/2, through the GTPase-activating domain of TSC2, drives the small GTPase Ras homolog enriched in brain (Rheb) into the inactive GDP bound state (Li et al., 2004). Recent studies indicate that GTP bound Rheb acts directly on the mammalian *t*arget *of r*apamycin (mTOR) (Long et al., 2005a) (Figure 1). mTOR is known to exist in an evolutionarily conserved, rapamycin-sensitive complex with two additional proteins, regulatory-associated protein of mTOR (raptor) and G protein β-subunit-like protein (GβL) (Hay and Sonenberg, 2004). It is presumably this complex (mTOR complex 1) that mediates S6K1 phosphorylation at a number of residues, as judged from in vitro studies (Isotani et al., 1999). These phosphorylation sites include T389, whose phosphorylation allows PDK1 to phosphorylate S6K1 T229 and activate the kinase (Alessi et al., 1998; Pullen et al., 1998). The ability of mTOR to mediate S6K1 activation is controlled by raptor, which recognizes S6K1 through its amino-terminal TOR signaling (TOS) motif (Schalm and Blenis, 2002). However, recent studies show that mTOR also exists in a second rapamycin-resistant signaling complex with GβL and a protein termed AVO3, or rapamycin-insensitive companion of mTOR (rictor), rather than raptor, and that this complex mediates the phosphorylation of S473, the equivalent site to S6K1 T389. In concert with PDK1 phosphorylation of T308, this leads to PKB activation (Sarbassov et al., 2005) (Figure 1). Thus, the role of the mTOR/rictor/GβL complex (mTOR complex 2) in PKB activation places it as a key upstream effector in S6K1 activation (Figure 1).

Amino acids and glucose

It has become evident over the last few years that mitogens and hormones, such as insulin, alone are insufficient to drive S6K1 activation in the absence of nutrients such as amino acids and glucose (Montagne and Thomas, 2004). These observations indicate that the insulin-induced signaling pathway is integrated with inputs from ambient nutrients to regulate mTOR complex 1 function and control S6K1 activation. The importance of nutrients in S6K1 signaling was first hinted at from studies in macroautophagy, a process in which portions of cytoplasm are sequestered into autophagosomes for transport to lysosomes for degradation and recycling of cellular constituents (Klionsky and Emr, 2000). In this way macroautophagy plays a critical role in maintaining cellular metabolic homeostasis in response to nutrient deprivation. Initially, Meijer and colleagues demonstrated that inhibition of macroautophagy by amino acid addition was paralleled by an increase in S6 phosphorylation, which they attributed to S6K1 activation (Blommaart et al., 1995). Moreover, they showed that amino acid inhibition of macroautophagy was in part suppressed by rapamycin, a fungicide produced by *Streptomyces hygroscopicus* (Dennis and Thomas, 2002). Rapamycin acts by forming an inhibitory complex with the immunophilin, FK506 binding protein 12 (FKBP12), which binds to and disrupts mTOR complex 1 signaling (Hay and Sonenberg, 2004; Montagne and Thomas, 2004). Consistent with these findings, it was subsequently demonstrated that amino acid-dependent S6K1 activation is mTOR dependent (Hara et al., 1998; Iiboshi et al., 1999), with a number of studies pointing to the importance of branched-chain amino acids, especially leucine (Hay and Sonenberg, 2004; Kimball and Jefferson, 2004). However, it should be noted that recent reports suggest that there

may be mTOR-independent routes involved in amino acid-induced autophagy inhibition (see Codogno and Meijer, 2005).

Energy

mTOR complex 1 signaling to S6K1 is also sensitive to small changes in the physiological levels of ATP, independent of alterations in amino acid levels (Dennis et al., 2001). Here, treatment with the mitochondrial complex 1 inhibitor rotenone was shown to induce a small reduction in intracellular ATP levels and, in parallel, lead to a strong attenuation of insulin-induced S6K1 activation (Dennis et al., 2001). Consistent with the proposal that homeostatic levels of ATP directly regulate mTOR signaling (Dennis et al., 2001), mTOR's apparent Km for ATP is similar to intracellular ATP concentrations (Gribble et al., 2000). However, subsequent studies reported that energy depletion inhibited mTOR signaling through AMP-activated kinase (AMPK) phosphorylation of TSC2 (Corradetti et al., 2004; Inoki et al., 2003; Shaw et al., 2004) (Figure 1). This observation would seem to support a pivotal role for AMPK in switching between energy-consuming and energy-sparing processes by sensing changes in the intracellular AMP/ATP ratio (Figure 1) (Carling, 2004). However, it is not known how AMPK phosphorylation of TSC2 enhances the ability of TSC1/2 to inhibit downstream signaling to Rheb (Inoki et al., 2003). Indeed, this site is not conserved in the *Drosophila* homolog of TSC2, dTSC2 (Ito and Rubin, 1999). Moreover, the role of AMPK has been placed into question by recent studies showing that the hypoxia-inducible gene, regulated in development and damage responses (REDD1), is also induced by energy depletion and that this leads to inhibition of mTOR complex 1 signaling to S6K1, but in a manner independent of AMPK (Sofer et al., 2005) (Figure 1). In support of this notion, absence of REDD1 does not alter AMPK activation, or its apparent ability to phosphorylate TSC2; however it ablates the inhibitory effects of energy depletion on mTOR complex 1 signaling to S6K1 (Sofer et al., 2005). The mechanism by which REDD1 mediates the effects of energy depletion on mTOR complex 1 signaling to S6K1 is unknown, but it appears to require TSC2 (Sofer et al., 2005). It should also be noted that the role of TSC1/2 in regulating the energy input to mTOR complex 1 and S6K1 activation has been challenged by Smith et al., who found that treatment of TSC2-deficient cells with 2-deoxyglucose still led to the inactivation of S6K1 (Smith et al., 2005). These findings raise the possibility that AMPK phosphorylation of TSC2 is not necessary for responses to energy challenge and that additional mechanisms may be implicated (see below).

Negative feedback loop

In parallel studies, it has become increasingly evident that S6K1 may also be implicated in a negative feedback loop to suppress insulin signaling. Such a role for S6K1 was first supported by the observation that amino acids inhibit insulin-induced PI3K signaling (Patti, 1999), a response later shown to be blunted by rapamycin (Tremblay and Marette, 2001). In parallel, genetic and biochemical studies in *Drosophila* showed that the S6K1/2 ortholog, dS6K, is a negative effector of dPKB (Radimerski et al., 2002a, 2002b). As larval growth is exquisitely sensitive to nutrients (Oldham et al., 2000), this raised the possibility that the negative effects of amino acids on insulin signaling may be mediated by S6K1 (Radimerski et al., 2002a). Consistent with these findings, *S6K1*$^{-/-}$ mice maintained on a high-fat diet (HFD)

remain insulin sensitive, as does insulin-induced PKB activation, despite the fact that insulin receptors desensitize in such mice (Um et al., 2004). That this response is due to loss of S6K1 is shown by the finding that siRNA knockdown of S6K1 protein in cells has no effect on insulin-induced receptor tyrosine auto-phosphorylation but potentiates insulin-induced PKB phosphorylation (Um et al., 2004). These studies suggested that S6K1 elicits a selective inhibitory effect on PKB activation at a point downstream of the insulin receptor. Further analyses revealed that phosphorylation of IRS1 S307 and S632 was reduced in $S6K1^{-/-}$ mice on a high-fat diet and in S6K1 siRNA-treated cells (Um et al., 2004), sites known to be elevated in animal models of obesity and in muscle from type 2 diabetics (Bouzakri et al., 2003). Moreover, whereas PKB activity was blunted in animals fed a HFD and in two genetic mouse models of obesity, S6K1 activity was highly elevated (Um et al., 2004). These findings suggested that S6K1 may positively affect IRS1 serine phosphorylation, leading to inhibition of PKB signaling and insulin resistance (Figure 3). This hypothesis was substantiated by results demonstrating in $TSC2^{-/-}$ mouse embryonic fibroblasts (MEFs), which exhibit constitutive S6K1 activation, that IRS1 is hyperphosphorylated and degraded (Harrington et al., 2004; Shah et al., 2004). These studies revealed that IRS1 S302, which is proximal to the IRS1 phosphotyrosine binding (PTB) domain and contains an S6K1 recognition motif (Flotow and Thomas, 1992), is phosphorylated by S6K1 (Harrington et al., 2004) (Figure 3). Moreover, evidence was provided that phosphorylation of this site disrupts the ability of the PTB domain to interact with the activated IR, which would lead to decreased insulin signaling (Harrington et al., 2004). These findings support the model that S6K1 mediates IRS1 serine phosphorylation, disrupting its interaction with IR and leading to its degradation. Degradation of phosphorylated IRS1 is mediated by its association with a 14-3-3 family member, which relocates IRS1 from low-density microsomes to the cytosol (Figure 3), where it can be accessed and degraded by the 26S proteasome (Craparo et al., 1997). However, it should be noted that others have reported that both IRS1 S302 and S307 phosphorylation are necessary, but not sufficient, for mediation of this response (Werner et al., 2004), suggesting that additional phosphorylation sites are involved. It will be of interest to determine whether in $S6K1^{-/-}$ mice phosphorylation of IRS1 S302 is abrogated and whether phosphorylation at this site is involved in a predisposition for insulin resistance in vivo.

In parallel, the Lamb and Hunter groups also demonstrated that in $TSC2^{-/-}$ cells IRS1 mRNA levels were reduced and restored by rapamycin treatment, with the latter effect blocked by actinomycin D (Harrington et al., 2004; Shah et al., 2004). Furthermore, Harrington et al. have shown that suppression of S6K1 mimics the effect of rapamycin treatment and restores IRS1 mRNA levels (Harrington et al., 2004). This raises the question of whether IRS1 is the only target of mTOR, through S6K1, by which mTOR contributes to the negative feedback loop to downregulate PKB. Shah et al. have shown that IRS2 protein levels are also reduced in $TSC2^{-/-}$ cells (Shah et al., 2004). Consistent with this finding, Rhodes and colleagues have recently shown that chronic activation of mTOR signaling by glucose and IGF-1 or adenoviral-mediated expression of a constitutively activated mTOR construct increases serine/threonine phosphorylation of IRS2 and its degradation by the proteasome, an effect blocked by rapamycin or lactacystin (Briaud et al.,

2005). Thus, in delineating the role of S6K1 in the regulation of insulin signaling it will be necessary to establish the extent to which IRS1 and 2 contribute to this response.

A novel nutrient signaling pathway

The emerging paradigm by which nutrient overload can lead to insulin resistance though activation of mTOR complex 1 signaling to S6K1 (Patti and Kahn, 2004; Um et al., 2004) has underscored the importance of understanding where nutrients enter the mTOR complex 1 signaling pathway. Contrary to earlier models, which suggested that nutrients acted through TSC1/2, recent findings support a model in which nutrients act on mTOR complex 1 signaling, independently of TSC1/2 (Nobukuni et al., 2005; Smith et al., 2005). Moreover, as Rheb-GTP levels do not drop in $TSC1^{-/-}$ or $TSC2^{-/-}$ cells following amino acid withdrawal (Nobukuni et al., 2005; Roccio et al., 2006), although S6K1 is inactivated, it seemed likely that the major amino acid input that mediated S6K1 activation resided on a pathway parallel to that of the TSC1/2-Rheb signaling axis (Nobukuni et al., 2005). Although it has been shown that amino acid withdrawal can lead to a transient drop in Rheb-GTP levels in some cell types, S6K1 remains inactivated (Roccio et al., 2006). Earlier studies demonstrated that wortmannin, a potent class 1 PI3K inhibitor, blocks amino acid-induced mTOR complex 1 signaling, as judged by S6K1 activation (Hara et al., 1998; Iiboshi et al., 1999). Given that amino acids do not induce PKB activation (Hara et al., 1998; Nobukuni et al., 2005), this implies that the wortmannin-sensitive amino acid input to mTOR complex 1 is not via inhibition of class 1 PI3K. Consistent with these arguments, siRNA depletion of class 1 PI3K blocked insulin-induced S6K1 activation but had no effect on amino acid-mediated S6K1 activation (Nobukuni et al., 2005). These observations led to the finding that the wortmannin-sensitive target is class 3 PI3K or human class III phosphatidylinositol 3-kinase, hVps34 (Figures 1 and 3), as siRNA knockdown of hVps34 blocks amino acid- and insulin-induced S6K1 activation but has no effect on PKB activation (Byfield et al., 2005; Nobukuni et al., 2005). Moreover, stimulation of cells with amino acids increases hVps34 activity and the production of phosphatidylinositol 3-phosphate (PI3P), the product of hVps34 (Byfield et al., 2005; Nobukuni et al., 2005), a response that is not altered in $TSC2^{-/-}$ cells, consistent with the state of S6K1 activation (Nobukuni et al., 2005) (Figures 1 and 3). PI3P mediates the recruitment of proteins containing Fab1/YOTB/ZK632.12/Vac1/EEA1 (FYVE) or PI3P-targeting-phox homology (PX) domains to endosomal membranes (Lemmon, 2003), with PI3P-rich microdomains recently having been shown to act as signaling platforms (Burda et al., 2002; Gonzalez-Gaitan and Stenmark, 2003; Miaczynska et al., 2004). Consistent with the role of hVps34 in mediating the amino acid input that leads to S6K1 activation, this response is blocked by the overexpression of a dominant interfering cDNA containing two FYVE domains (Nobukuni et al., 2005). Interestingly, Backer and colleagues have also shown that energy as well as osmotic stress regulate hVps34 activity in a manner similar to that of amino acid withdrawal, suggesting that hVps34 may represent a common nutrient sensor for S6K1 activation (Byfield et al., 2005).

The question that arises from these studies concerns the identity of the mechanism by which hVps34 exerts these effects and how they are integrated with those of the insulin signaling pathway to mediate S6K1 activation. It has been shown that either amino acid or glucose withdrawal drives the mTOR

complex 1 into a so-called "inactive conformation," which is measured by the stronger binding of raptor to mTOR and the inability of the complex to phosphorylate S6K1 in vitro (Kim et al., 2002). In contrast, re-addition of amino acids reverts this complex into a so-called "active conformation," reflected by a weaker interaction of raptor with mTOR and the restored ability to signal to S6K1 in vitro (Kim et al., 2002). It has also been shown that in contrast to raptor, Rheb binds to the mTOR catalytic domain and that its binding is reversibly inhibited by amino acid withdrawal, regardless of GTP or GDP loading (Long et al., 2005a, 2005b). Though highly speculative, it may be that hVps34 drives the mTOR complex 1 into an active conformation, allowing Rheb to bind. In summary, these most recent studies have provided a framework for investigating the underlying molecular signaling mechanisms by which the ancient nutrient effector mTOR complex 1 signaling pathway has been integrated with that of humoral or insulin signaling to control growth during the rise of metazoans (Figures 1 and 3).

S6K1$^{-/-}$ mice have impaired glucose homeostasis

A deeper understanding of the role of S6K1 as a nutrient effector originally arose in studies of mice in which the gene had been deleted (Shima et al., 1998). Such mice displayed a lean phenotype and were approximately 20% smaller at birth, an effect which was more pronounced during embryogenesis (Shima et al., 1998). Moreover, in all tissues examined in S6K1$^{-/-}$ mice, S6K2 transcripts were upregulated, although this did not rescue the impaired growth phenotype associated with S6K1$^{-/-}$ mice (Shima et al., 1998). Consistent with this finding, mice lacking both S6K1 and S6K2 are no more severely affected in growth than S6K1$^{-/-}$ mice, although they show a sharp reduction in viability due to perinatal lethality (Pende et al., 2004). In addition to S6 phosphorylation, proliferation as well as the translation of 5′ terminal oligopyrimidine (5′ TOP) mRNA were intact in S6K1$^{-/-}$ MEFs (Shima et al., 1998). The 5′ TOP mRNAs encode for components of the translational apparatus (Meyuhas et al., 1996). The fact that their expression was unaffected in S6K1$^{-/-}$ mice indicated that the small body size phenotype did not stem from reduction in the amount of translational machinery. The phenotype of these mice and the sensitivity of S6K1 activation to amino acids (Hara et al., 1998) and glucose (Hugl et al., 1998) led to the finding that S6K1$^{-/-}$ mice are mildly glucose intolerant and have reduced levels of circulating insulin (Pende et al., 2000). Reduced insulin levels result from a selective reduction in β cell volume, which has a pronounced effect on both pancreatic insulin content and insulin secretion (Pende et al., 2000) (Figure 2). These findings also suggest that the function of S6K1 in pancreatic β cell growth and insulin secretion is distinct from that of S6K2. Seemingly consistent with this finding, recent studies show that knockin mice, in which the wild-type allele of ribosomal protein S6 was replaced by a mutant having the five sites of serine phosphorylation changed to alanines, exhibit reduced pancreatic β cell size and glucose intolerance (Ruvinsky et al., 2005). However, despite the apparent similarity in phenotype between S6K1$^{-/-}$ mice and mutant S6 knockin mice, it is still unclear whether reduced pancreatic β cell size in S6K1$^{-/-}$ mice can be attributed to reduced S6 phosphorylation, as the major S6 kinase appears to be S6K2 in all tissues examined to date (Pende et al., 2004), including pancreas (M. Pende, S.H.U., and S.C. Kozma, unpublished data). In addition, it has yet to be determined whether reduced
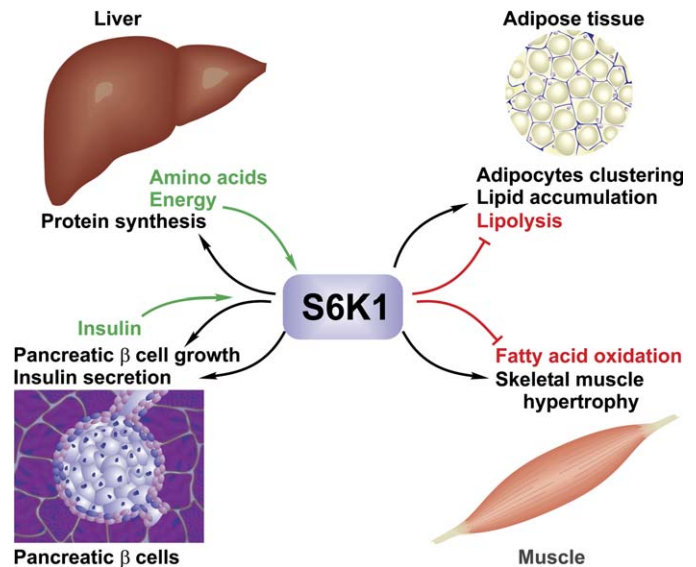

Figure 2. The role of S6K1 in nutrient- and insulin-responsive tissues

Insulin, amino acids, and energy activation of S6K1 signaling induce anabolism, including lipid accumulation in adipose tissue, skeletal muscle hypertrophy, pancreatic β cell growth, and protein synthesis. S6K1 signaling also suppresses catabolic events such as lipolysis in adipose tissue and fatty acid oxidation in muscle, both of which generate ATP.

pancreatic β cell size in S6K1-deficient mice, or in S6 knockin mice, is due to a cell-autonomous defect.

Despite the fact that S6K1$^{-/-}$ mice are mildly glucose intolerant and display hypoinsulinemia, they maintain normal fasting and feeding glucose levels in the fasting and fed states, which initially led to the hypothesis that such mice may be more sensitive to insulin in their peripheral tissues than wild-type mice (Pende et al., 2000). This speculation eventually led to the finding of the negative feedback loop whereby insulin-induced S6K1 activation reduced insulin signaling (Um et al., 2004). In the absence of this signaling regulation, S6K1$^{-/-}$ mice have enhanced insulin sensitivity as a function of either age or diet (Um et al., 2004). This distinct phenotype, hypoinsulinemia coupled with increased insulin sensitivity, raised the question of whether such mice would be protected against diet-induced obesity (Um et al., 2004). In fact, when S6K1$^{-/-}$ mice were placed on HFD, the rate of weight accumulation was dramatically reduced as compared to wild-type mice, consistent with MRI analyses demonstrating a striking reduction in fat depots (Um et al., 2004) (Figure 2). Assessment of total fat depots revealed that the body fat index for S6K1$^{-/-}$ mice increased by only 15%–20% over this period whereas the value for wild-type mice doubled (Um et al., 2004). This decrease in fat accumulation appears to be due to a dramatic rise in lipolysis in adipose and energy combustion in both adipose and muscle (Um et al., 2004). Thus, S6K1$^{-/-}$ mice are protected against diet-induced obesity due to a sharp increase in lipolysis and metabolic rate, which is linked to enhanced oxidative phosphorylation (Um et al., 2004).

Adipose tissue

That S6K1 plays an important anabolic role in insulin-responsive tissues has been revealed by specific phenotypes displayed by S6K1$^{-/-}$ mice. As stated above, S6K1$^{-/-}$ mice exhibit

increased lipolysis as well as an apparent reduction in numbers of preadipocytes and adipose tissue mass (Um et al., 2004). A link between S6K1 and adipogenesis was first suggested in studies that showed that rapamycin inhibits clonal expansion and adipocyte differentiation and in parallel inhibits the expression of CCAAT/enhancer binding protein α (C/EBPα), a transcription factor required for 3T3-L1 cell differentiation (Yeh et al., 1995). Following this initial observation it was found that rapamycin can also disrupt adipogenesis in primary human adipocytes independent of its antiproliferative effect on clonal expansion (Bell et al., 2000) since primary human adipocytes undergo differentiation in the absence of clonal expansion. In support of this observation, it was recently reported that the inhibitory effect of rapamycin persists throughout the process of adipocyte differentiation (Cho et al., 2004). This significantly reduces expression of most adipocyte marker genes, including Peroxisome Proliferator-Activated Receptor γ (PPARγ) and Fatty Acid Synthase (FAS), and decreases intracellular lipid accumulation (Cho et al., 2004). Interestingly, it was shown that 3T3-L1 adipocytes subjected to amino acid deprivation, like rapamycin treatment, suppresses the induction of C/EBPα, providing a potential link between nutrients and adipogenesis transcriptional programs. Both amino acid deprivation and rapamycin treatment specifically disrupt the positive transcriptional feedback loop between PPARγ and C/EBPα, two key transcription factors involved in mediating adipogenesis by directly targeting the transactivation activity of PPARγ (Kim and Chen, 2004). Taken together, these findings would support a model whereby reduced adipogenesis in response to rapamycin treatment may arise from impaired S6K1 signaling. Such impairment could be induced by one of two adipogenic stimuli, fatty acids and/or PPARγ agonists, and amino acid availability. However, whereas a rapamycin-resistant mutant of mTOR conferred rapamycin resistance on both adipogenesis and C/EBPα induction, neither a rapamycin-resistant mTOR kinase dead mutant nor a rapamycin-resistant S6K1 mutant conferred rapamycin resistance on either response (Kim and Chen, 2004). These studies could be interpreted in two ways. First, as the rapamycin-resistant S6K1 mutant is presumably regulated by mTOR complex 2 rather than mTOR complex 1 (Ali and Sabatini, 2005), one possibility is that this construct may not faithfully mimic wild-type S6K1 function. On the other hand, the effects observed on adipogenesis in S6K1$^{-/-}$ mice may be due to developmental or humoral stimuli, such that the effects observed on adipogenesis are not attributable to a cell-autonomous effect on adipocytes. It will be important to resolve such issues in future studies.

Muscle

Like β cells and adipocytes, muscle growth, or hypertrophy, also appears to be strongly affected in S6K1$^{-/-}$ mice. Hypertrophy of muscle is characterized by an increase in the size of myofibers, which can occur as an adaptive response to load-bearing exercise. PKB and mTOR signaling are upregulated during muscle hypertrophy and downregulated during muscle atrophy (Bodine et al., 2001). Consistent with this, hypertrophy in cell culture and in compensatory animal models is blocked by rapamycin, and these inhibitory effects appear to be mediated by inhibition of S6K1 (Ohanna et al., 2005) (Figure 2). This conclusion is based on recent results of Pende and colleagues, who showed that S6K1$^{-/-}$ mice exhibit an atrophy-like phenotype in skeletal muscle without having altered PKB or eIF4E binding
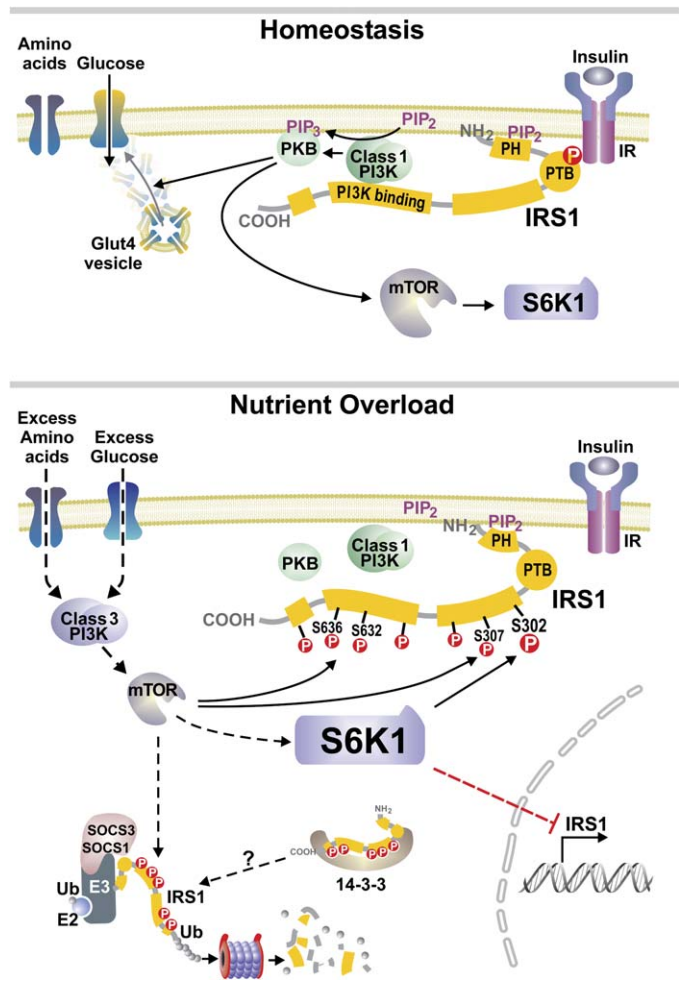

Figure 3. Homeostasis versus nutrient overload

In a homeostatic setting, as nutrients and amino acids are consumed, mTOR/S6K1 activity participates in mediating cell growth. In contrast, under conditions of nutrient overload associated with the obese state, constitutive activation of mTOR/S6K1 signaling leads to desensitization of insulin signaling through IRS1 serine phosphorylation and inhibition of IRS1 transcription. Serine phosphorylation of IRS1 associates with a 14-3-3 family member, localizes IRS1 to cytosol, and subsequently leads to proteasomal degradation. IRS1 also associates suppressor of cytokine signaling-1 (SOCS1) and SOCS3, which form an E3 ubiquitin ligase complex leading to the proteasome degradation pathway.

proteins 1/2 (4E-BP1/2) activation (Ohanna et al., 2005). Moreover, they further demonstrated that S6K1 mediates the IGF1-induced skeletal muscle hypertrophy phenotype, which is dependent on mTOR but does not affect muscle cell-cycle progression induced by constitutively activated PKB (Ohanna et al., 2005). Similar results have been obtained in C2C12 cells (Park and Chen, 2005). The fact that the impaired hypertrophic response to IGF1 in S6K1$^{-/-}$ cells does not correlate with S6 phosphorylation or overexpression of S6K2 would argue for a novel unidentified S6K1 substrate (Ohanna et al., 2005). Interestingly, Richardson et al. have shown that the S6K1 Aly/REF-like target (SKAR), an RNA binding protein, is a specific target for S6K1, but not for S6K2, in controlling cell growth (Richardson et al., 2004). However, whether SKAR is involved in skeletal muscle hypertrophy has not been studied.

S6K1 signaling in metabolic disease

The importance of nutrient overload in type 2 diabetes is strongly supported by recent intervention trials showing that weight loss through exercise or dietary modifications can reduce the incidence of type 2 diabetes by 60% (Knowler et al., 2002; Tuomilehto et al., 2001). As plasma concentrations of amino acids are increased in obesity (Felig et al., 1969, 1970, 1974) and amino acids drive S6K1 activation (Hara et al., 1998; Iiboshi et al., 1999; Nobukuni et al., 2005), it is hypothesized that insulin resistance in obese humans may in part be mediated by S6K1 phosphorylation of IRS1 (Um et al., 2004) (Figure 3). To address the metabolic mechanisms by which elevated amounts of amino acids lead to insulin resistance, the effects of amino acid infusion in humans has been studied in both liver and muscle through the use of $^{31}P/^{13}C$ nuclear magnetic resonance spectroscopy (Krebs et al., 2002, 2003). In liver, increased amounts of circulating amino acids were found to increase gluconeogenesis (Krebs et al., 2003). This increase was found to be independent of the ability of amino acids to induce the secretion of glucagon and insulin but primarily due to the fact that they serve as substrates to drive gluconeogenesis. Although the amino acid-induced increase in insulin secretion protects against hyperglycemia induced by endogenous glucose production in healthy individuals, impaired insulin secretion unmasks the contribution of amino acids through increased gluconeogenesis (Krebs et al., 2003). Although S6K1 is highly phosphorylated in livers of obese rodents, and this effect as well as insulin resistance are reversed by the administration of rapamycin (Ueno et al., 2005), it is not known whether amino acids contribute directly to hepatic insulin resistance through S6K1 activation (Figure 2).

In skeletal muscle, insulin stimulation leads to the uptake of glucose, through PKB-mediated translocation of the glucose transporter-4 to the cell surface (Figure 3). Like free fatty acids, many amino acids are metabolized to acetyl-coenzyme A and oxidized in mitochondria. In this way amino acids might be speculated to compete with glucose as an energy source at the level of β oxidation in mitochondria, leading to hyperglycemia (see Krebs, 2005). Consistent with this model, an approximate 2-fold rise in serum plasma amino acid concentrations is paralleled by an approximately 25% reduction in insulin sensitivity (Krebs et al., 2002). However, the reduction in insulin sensitivity is preceded by a decrease in the rise of intramuscular glucose-6 phosphate and followed by a sharp drop in glycogen synthesis, arguing that the amino acids are not competing as substrates but are directly inhibiting glucose transport and phosphorylation (Krebs et al., 2002). Compatible with this data and those in mice (Um et al., 2004), recent studies in humans have also shown that the amino acid infusion-induced inhibition of insulin-stimulated glucose disposal in skeletal muscle is paralleled by IRS1 phosphorylation at S312 and S636/639 (S307 and S632/635 in the mouse) and an inhibition of insulin-induced PI3K activation (Tremblay et al., 2005) (Figure 3). Therefore, excess amino acids contribute to insulin resistance by inhibiting glucose uptake, one of the earliest defects responsible for the development of type 2 diabetes (Martin et al., 1992). Moreover, impaired glucose uptake appears to be mediated through phosphorylation of IRS1 by S6K1 (Tremblay et al., 2005), a system that may have evolved to suppress insulin signaling under conditions of nutrient overload (Figure 3). Future studies will determine whether deregulation of the nutrient signaling pathway is due to genetic alter-ations that predispose to the early onset of obesity, and whether insulin resistance and/or excess nutrient intake cause constitutive activation of this pathway leading to desensitization of class 1 PI3K-dependent insulin signaling in individuals.

In conclusion, understanding the mechanisms by which amino acids impact on the nutrient-sensitive signaling pathways through such molecules as hVps34, leading to S6K1 activation, may provide novel strategies for the treatment of insulin resistance.

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