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Amino Acid Regulation of Autophagosome Formation

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Summary

Amino acids are not only substrates for various metabolic pathways, but can also serve as signaling molecules controlling signal transduction pathways. One of these signaling pathways is mTOR-dependent and is activated by amino acids (leucine in particular) in synergy with insulin. Activation of this pathway inhibits autophagy. Because activation of mTOR-mediated signaling also stimulates protein synthesis, it appears that protein synthesis and autophagic protein degradation are reciprocally controlled by the same signaling pathway.

Recent developments indicate that amino acid–stimulated mTOR-dependent signaling is subject to complex regulation. The mechanism by which amino acids stimulate mTOR-dependent signaling (and other signaling pathways), and its molecular connection with the autophagic machinery, is still unknown.

Key Words: Amino acid signaling; insulin; phosphatidylinositol 3-kinase (PI3K); mammalian target of rapamycin (mTOR); ribosomal protein S6; AMP-activated protein kinase (AMPK); AICAriboside (AICAR); glutamate dehydrogenase; beclin 1; reactive oxygen species.

1. Introduction

Cells adapt to changes in their environment by adjusting anabolic and catabolic pathways. In times of nutrient shortage, for example, macromolecules are degraded to produce substrates for energy production. Macroautophagy ("autophagy") is the major, lysosomal mechanism by which cells degrade protein, in addition to the multienzyme proteasome system. Unlike the proteasome, however, autophagy can also eliminate damaged or redundant organelles.

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During autophagy, part of the cytoplasm is sequestered by a double membrane, the origin of which is still not firmly established but which is presumably derived from specialized regions of the endoplasmic reticulum ***(1)***. Several Atg proteins, first discovered in yeast, are involved in the formation and maturation of autophagosomes ***(2)***. Mammalian counterparts of most of the ATG genes have also been found ***(2)***.

After their formation, the initial autophagosomes acquire their lytic enzymes by fusion with existing or newly formed lysosomes to form autophagolysosomes upon which degradation of the sequestered material can occur.

When the cellular nutrient supply becomes insufficient, autophagy is activated. A classical example is that of the mammalian liver, which, in starvation, degrades proteins by autophagy in order to produce amino acids for gluconeogenesis, glucose being required for the brain and the erythrocytes. However, even under nutrient-rich conditions, some ongoing autophagy is still required in order to allow cells to remove defective cell structures. Thus, mice with specific knock-outs of Atg5 or Atg7 in neuronal cells develop neurodegeneration because of defective autophagy ***(3,4)***.

2. Control of Autophagy

For decades, amino acids have been known as (product) inhibitors of autophagy and they do so by inhibition of autophagosome formation ***(5)***, although an effect of some amino acids on autophagosome fusion with lysosomes and on the intralysosomal pH (leucine) (*see* **ref. *6*** for literature) cannot be ruled out. In addition, autophagy has long been known to be inhibited by insulin and promoted by glucagon ***(5,7)***.

The mechanism by which amino acids inhibit autophagosome formation has been obscure for a long time. However, research carried out in our own and other laboratories over the last 10–15 years has indicated that amino acids can control autophagy via changes in the activity of signal transduction pathways. Among these, the mTOR-dependent signal transduction pathway, also used by insulin and other growth factors, appears to play a prominent role ***(8)***. However, other signaling pathways, such as the ras/raf/Erk1/2 pathway ***(9)*** and the integrin/$p38^{MAPK}$ pathway, may also be affected ***(10)***.

2.1. Insulin, Amino Acids, mTOR-Mediated Signaling, and Autophagy

Before discussing the stimulation of mTOR-dependent signaling by amino acids, and its relationship with autophagy, a short description of the insulin signaling pathway is given **(Fig. 1)** (*see* **refs. *11*** and ***12*** for a detailed discussion of the various components in this pathway).

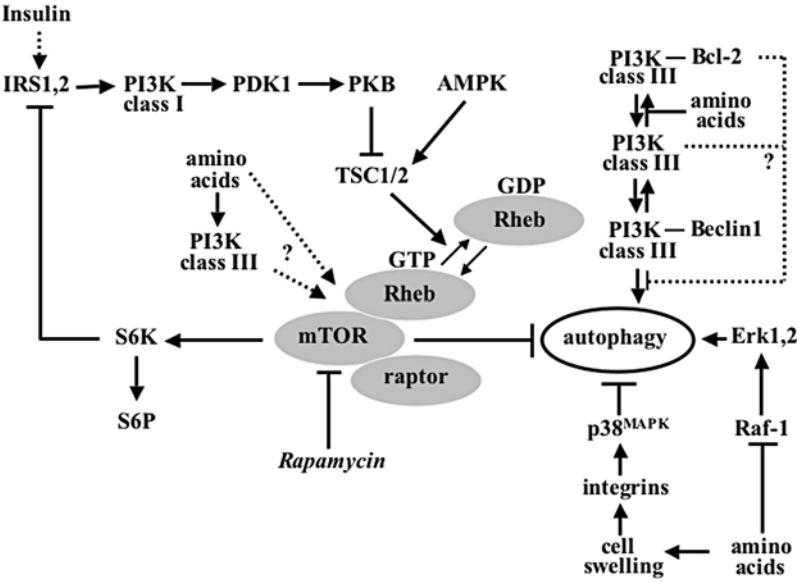

Fig. 1. Amino acid–activated signaling and the regulation of autophagy. Abbreviations: IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase class; PDK1, phosphoinositide-dependent kinase-1; PKB, protein kinase B; AMPK, AMP-activated protein kinase; TSC, tuberous sclerosis complex; Rheb, Ras homolog enriched in brain; raptor, regulatory associated protein of mTOR; mTOR, mammalian target of rapamycin; S6K, 70 kD S6 kinase; S6, ribosomal protein S6; MAPK, mitogen-activated protein kinase; Erk, extracellular regulated protein kinase.

The first part of the insulin-dependent signal transduction pathway is located upstream of the protein kinase mTOR and includes the insulin receptor, IRS1 and 2, PI3K class I (producing PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2) and protein kinase B. This part is involved in the the regulation of carbohydrate metabolism ***(13)***.

The second part of the insulin-signaling pathway, located downstream of mTOR, includes components such as S6K, 4E-BP1, ribosomal protein S6, eIF2α-kinase, and eEF-2 kinase, proteins that are involved in the regulation of protein synthesis ***(12)***. mTOR activity is controlled by the heterodimer TSC1/TSC2, which acts as a brake on mTOR-dependent signaling. TSC1/TSC2 acts as a GTPase-activating protein complex for the small G-protein Rheb, which, in its GTP form binds to and activates mTOR ***(12,14)***. When TSC2 is phosphorylated by protein kinase B, the TSC1/TSC2 complex becomes inactive and mTOR signaling activated.

mTOR is present in a complex with raptor, a protein that functions as a scaffold for mTOR-mediated phosphorylation of mTOR substrates; the protein GβL is also part of this complex ***(15)***. In this form, mTOR activity is inhibited by rapamycin. When, however, mTOR is complexed with another protein, rictor, its kinase activity is rapamycin-insensitive and serves to control protein kinase B phosphorylation ***(15)***.

Early experiments with protein phosphatase inhibitors in isolated hepatocytes had already indicated that protein phosphorylation was involved in the control of autophagy, but the link with amino acids was not made in these studies ***(16)***.

A breakthrough in the search for mechanisms by which amino acids inhibit autophagy (and stimulate protein synthesis) was obtained in studies with [^{32}Pi]phosphate-labeled hepatocytes carried out in our laboratory. In these studies, we discovered that the same amino acids that inhibited autophagy, rapidly (within 20 min) and greatly (up to fivefold) stimulated the phosphorylation of a 31 kDa ribosomal protein that we identified as S6 ***(17,18)***.

Under a variety of experimental conditions, with various amino acid mixtures, in the absence and presence of insulin and/or glucagon, there was a linear relationship between the percentage inhibition of autophagic proteolysis (measured in the presence of low concentrations of cycloheximide to inhibit simultaneous protein synthesis) and the degree of S6 phosphorylation. Insulin and low concentrations of amino acids acted in synergy: insulin inhibited autophagy and stimulated S6 phosphorylation, but only in the presence of low amino acid concentrations, not in the absence of amino acids (when autophagy was maximal) or at high amino acid concentrations (when autophagy was already minimal). Glucagon had the opposite effect: it stimulated autophagy but only in the presence of low amino acid concentrations, not in the absence of amino acids or in the presence of high amino acid concentrations. Among the various amino acids that inhibit autophagy, leucine (but not valine) was particularly effective in inhibiting autophagy and stimulating S6 phosphorylation.

Amino acid–induced S6 phosphorylation was completely inhibited by rapamycin, indicating that mTOR and S6K were components of the signaling pathway **(Fig. 1)**. Of great significance was the fact that rapamycin could partly, albeit not completely, reverse the inhibition of autophagy by amino acids. Because the mTOR pathway was known to be involved in the regulation of protein synthesis, we concluded that, apparently, protein synthesis and (autophagic) protein degradation are controlled by the same signaling pathway, which is efficient from the point of view of metabolic regulation ***(18)***. The observation that rapamycin increases autophagy has been confirmed for many other cell types ***(19)***, including yeast ***(20,21)***.

Our studies were important for two reasons. First, they were the first to show that amino acids can act as signaling molecules that stimulate a signal

transduction pathway sharing components with the insulin signaling pathway, with wide ramifications for the regulation of metabolism, not only for protein synthesis and autophagy, but, as we will see later, also for carbohydrate metabolism. Second, they suggested a possible mechanism for the inhibition of autophagy by amino acids.

Since our initial observations, the ability of amino acids to stimulate mTOR-dependent signaling and the synergy between insulin and amino acids have been confirmed (or rediscovered) for many other insulin-sensitive cell types, with leucine being the most effective amino acid (*see* **refs. *8*** and ***15*** for reviews). Likewise, the inhibition of mTOR signaling by glucagon has been confirmed ***(22,23)***.

As indicated above, an important feature of amino acid signaling in hepatocytes is that high concentrations of amino acids alone are sufficient to stimulate mTOR-dependent signaling even when insulin is not present ***(8,18)***. Conversely, the effect of insulin on mTOR-dependent signaling is potentiated by the presence of low concentrations of amino acids and is absent when amino acids are severely depleted. In contrast to insulin, amino acids do not activate protein kinase B and probably also not PI3K class I (*see* **ref. *8*** for review). Because amino acid–stimulated mTOR-dependent signaling is prevented by inhibitors of PI3K, as was first shown by our laboratory ***(24)*** and later by others ***(8,15)***, it is likely that mTOR receives two parallel inputs, one via PI3K class I / PDK1 / PKB / TSC1,2 / Rheb and one via amino acids (*see* **Fig. 1**). Thus, both PI3K class I (by insulin) and mTOR (by amino acids) must be activated to ensure full activation of mTOR downstream targets. Because high concentrations of amino acids are sufficient to stimulate mTOR signaling maximally, one has to assume that basal activity of PI3K class I is sufficient for mTOR-dependent signaling under these conditions ***(25)***. Recent information suggests that amino acids may not stimulate PI3K class I but rather PI3K class III, which produces PtdIns(3)P that directly stimulates mTOR without the requirement for protein kinase B ***(15,26,27)*** (*see also* below).

Another development which was important for a better understanding of the regulation of autophagy was the finding that interruption of signaling by inhibitors of PI3K class I (wortmannin, LY294002), in contrast to that caused by rapamycin, did not stimulate but rather inhibited autophagy ***(24)***. In order to account for this unexpected result we hypothesized that PtdIns(3)P, the product of PI3K class III, is essential for autophagosome formation whereas PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, the products of PI3K class I, are inhibitory and that the PI3K inhibitors are not entirely specific and inhibit both PI3K class I and III ***(6)*** (*see* **Fig. 1**); the basis of this hypothesis was the situation in yeast cells which are very active in autophagy under nutrient-deprived conditions but only contain a PI3K class III analog, Vps34, but

not a PI3K class I. The hypothesis was tested in HT-29 cells and appeared to be correct: PtdIns(3)P appeared to be essential for autophagy, whereas PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 were inhibitory ***(28)***. In addition, overexpression of PTEN, which hydrolyzes PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 and PtdIns(3,4)P_2 to PtdIns(4)P, stimulated autophagy in human colon cancer HT-29 cells ***(29)***. 3-Methyladenine, the classical inhibitor of autophagy ***(30)***, turned out to be a PI3K inhibitor; inhibition of PtdIns(3)P formation provided a satisfatory explanation for its mechanism of action ***(24,28)***.

Interestingly, Beclin1, the mammalian homolog of Atg6, which is involved in autophagosome formation ***(31),*** is found in a complex with PI3K class III ***(32,33)***. In order to be able to bind to PI3K class III and to stimulate autophagy, Beclin 1, which is found in association with the anti-apoptotic protein Bcl-2, must first dissociate from the inhibitory Beclin 1-Bcl-2 complex ***(34)***.

In addition to their ability to inhibit autophagy via mTOR activation, amino acids also inhibit autophagy by decreasing Beclin1-associated PI3K class III activity ***(35)***, because of increased binding of Beclin 1 by Bcl-2 ***(34)***. Presumably, this accounts for the observation that inhibition of autophagy by amino acids is rapamycin-insensitive under some conditions ***(36–38)***.

At variance with a requirement of autophagy for the Beclin 1-PI3K class III complex are recent studies showing that amino acids stimulate mTOR signaling via activation of PI3K class III and that amino acid depletion results in a decrease in the activity, but not the amount, of Beclin 1-associated class III PI3K ***(15,26,27)***. In these studies, autophagy was not investigated, however. A possible explanation is that there are different pools of PI3K class III, differentially regulating autophagy and mTOR signaling ***(15,26,27)***. Perhaps an excess of free PI3K class III, not bound to Beclin 1 (or bound to Bcl-2, *see* previous paragraph), signals to mTOR and inhibits the stimulation of autophagy by the Beclin 1-PI3K class III complex (*see* **Fig. 1**).

According to Hinault et al. ***(39)***, amino acids are able to stimulate the insulin-signaling pathway in yet another manner. These authors propose that amino acids can stimulate protein kinase B in a PI3K-independent manner because they stimulated protein kinase B phosphorylation in the presence of insulin and wortmannin ***(39)***. It must be pointed out, however, that amino acids can react chemically with wortmannin in a nonenzymic fashion and thus relieve the inhibition of PI3K ***(40)***.

Although the molecular connection between mTOR and the machinery required for autophagy is not yet firmly established, experiments with yeast have indicated that the Atg1–Atg13 complex, which is required for autophagosome formation, is one of the targets. Starvation of yeast cells, or rapamycin treatment, dephosphorylates Atg1 and enhances its protein kinase activity; Atg13, which binds to and activates Atg1, is hyperphosphorylated under

nutrient-rich conditions in a Tor-dependent manner, reducing its affinity for Atg1 ***(41)***. Recent evidence obtained with yeast indicates that the protein kinase activity of Atg1 is essential for autophagosome formation ***(42)***.

2.2. mTOR, AMP-Activated Protein Kinase, and Autophagy

In addition to its ability to sense amino acids, mTOR may also act as a sensor of the cellular energy state. Originally it was thought that mTOR senses the intracellular ATP concentration because of its relatively low affinity for ATP ***(43)***. However, later simultaneous observations in several laboratories, including our own, have indicated that small decreases in ATP result in activation of AMPK which, in turn, inhibits mTOR ***(44)*** and inhibits protein synthesis. This is in agreement with the function of AMPK to shut off ATP-dependent processes ***(45)***. AMPK possibly inhibits mTOR signaling by phosphorylating TSC2 ***(46)***.

In myotubes, amino acids stimulate phosphorylation of mTOR at Ser^{2448}. In the absence of amino acids, however, or by activation of AMPK, Thr^{2446} is phoshorylated. The close proximity of these two phosphorylation sites makes them mutually exclusive in that phosphorylation of one site inhibits that of the other and vice versa; they are viewed as switches integrating the opposing signals of growth factors and nutrient deprivation ***(47)***.

Inhibition of mTOR by AMPK, like that caused by addition of rapamycin may be expected to increase autophagy **(Fig. 1)**. However, there is controversy in the literature on this issue. In yeast, activation of AMPK, indeed, stimulates autophagy ***(48)***. By contrast, activation of AMPK by addition of the cell-permeable nucleotide analogue AICAriboside (AICAR) in hepatocytes strongly inhibits autophagy ***(49)***.

Recent evidence, however, indicates that also in mammalian cells, AMPK is required for autophagy. Thus, apoptotic stimuli, which result in increased mitochondrial permeability and decreased mitochondral membrane potential, target these mitochondria for autophagic degradation ***(50,51)***; it is likely that AMPK under these conditions is activated. Inhibition of mitochondrial ATP synthesis with oligomycin in insect cells has been shown to promote massive autophagy, of mitochondria in particular ***(52)***. In mouse embryonic fibroblasts, activation of the tumor suppressor p53 inhibits mTOR activity through activation of AMPK, a phenomenon that is accompanied by increased autophagy ***(53,54)***. Eukaryotic elongation factor 2-kinase (eEF-2 kinase) is essential for autophagy ***(55)*** and is known to be activated by AMPK ***(56)***. In a study recently carried out with different mammalian cell types, we obtained direct evidence that AMPK activation, as in yeast, promotes autophagy; we also concluded that the inhibition of autophagy by AICAR is unrelated to its ability to activate AMPK ***(57)***.

2.3. Mechanism of Amino Acid Sensing in mTOR-Mediated Signaling

Although amino acids can activate mTOR, the mechanism by which this occurs is unknown. Possible mechanisms are discussed below.

2.3.1. Amino Acid Receptor: Intra- or Extracellular?

Most evidence indicates that the amino acid receptor is intracellular rather than extracellular ***(58,59)***. It has been proposed that the plasma membrane contains a leucine-specific receptor protein that controls autophagy independent of mTOR ***(37)***; this receptor protein cannot be the leucine transporter as proposed ***(15)***, because both valine and isoleucine, which are transported via the same transporter, neither affect autophagy ***(6)*** nor mTOR-mediated signaling ***(60,61)***. It cannot be excluded that both intra- and extracellular sensing mechanisms operate in parallel or that their relative activity is cell-type–dependent.

One mechanism for intracellular amino acid sensing is that amino acids inhibit TSC1/2. Recent evidence indicates that at least TSC2 is not involved ***(62)***. Because AMPK activation inhibits mTOR signaling by phosphorylating TSC2 (*see* **Subheading 2.2.**), the possibility that amino acids stimulate mTOR by inhibition of AMPK can also be ruled out.

Another recent study has indicated that amino acids promote the association of Rheb with mTOR, an effect that is probably not caused by increased charging of Rheb with GTP ***(63)*** (contrast **ref.** ***64***, where an increase in Rheb-GDP upon nutrient depletion was found) but rather by direct action on mTOR ***(65)***. Although it is tempting to conclude that mTOR itself is the amino acid sensor, it is still possible that the effect of amino acids is indirect and that they decrease the concentration of an inhibitor that interferes with the Rheb-mTOR association ***(65)***.

As indicated in **Subheading 2.1.**, the possibility that amino acids are able to stimulate mTOR signaling via activation of PI3K class III, at least under some conditions, cannot be ruled out. But how amino acids exert this effect remains to be elucidated.

2.3.2. Amino Acid Sensing via Glutamate Dehydrogenase?

In most cell types, leucine (but not the other branched-chain amino acids) is one of the most potent amino acids in stimulating mTOR-dependent signaling ***(8)***; metabolism of leucine is not required ***(66)***. In addition, nonmetabolizable analogues of leucine can mimic its effect ***(60,61)***. Leucine is also very potent in promoting the association between mTOR and Rheb ***(65)***. Interestingly, in pancreatic β-cells the specificity of leucine (e.g., valine and isoleucine are not effective) and its analogs in stimulating mTOR signaling is strikingly similar

to the ability of these amino acids to stimulate glutamate dehydrogenase, the enzyme which is thought to play an important role in insulin production in β-cells ***(67)***. A possible connection between glutamate dydrogenase and mTOR signaling may be the production of GTP via oxidation of α-oxoglutarate, formed by glutamate dehydrogenase, for GTP charging of Rheb. It must be pointed out, however, that this GTP is produced within the mitochondria and somehow must be transported to the cytosol, a function that cannot be carried out by the mitochondrial adenine nucleotide transporter ***(68,69)***. Because nucleosidediphosphate kinase is found both within the mitochondria ***(70)*** and at the mitochondrial outer membrane ***(71)***, the exit of GTP from the mitochondria may be indirect via transfer of its terminal phosphate to ADP within the mitochondria and channeled back to GDP at the mitochondrial outer membrane. As indicated above, there is some controversy on whether ***(64)*** or not ***(63)*** the charging of Rheb with GTP is increased by amino acids. In principle, this would be an attractive hypothesis, especially because in cancer cells glutamine is an important substrate for energy production ***(72)*** so that flux through glutamate dehydrogenase may be high, mTOR signaling is very active and autophagy suppressed in cancer cells ***(19)***.

Another hypothesis is that NADPH produced by glutamate dehydrogenase in the direction of deamination, via the glutathione redox system, is engaged in the scavenging of reactive oxygen species which may be involved in the initiation of autophagy, at least under some conditions ***(73,74)***.

2.3.3. Diadenosine Polyphosphates

An intriguing possibility, which has not been mentioned in the literature so far, is that diadenosine polyphosphates (ApnA), byproducts of the aminoacyl-tRNA synthetase reaction, are involved in mTOR stimulation. Indirect evidence comes from two independent studies: in one study, leucine stimulated Ap4A production by the mitochondria in pancreatic ß-cells ***(75)***, while in another study leucine stimulated mTOR activity in ß-cells through increased mitochondrial oxidative metabolism ***(67)***, as discussed in the previous section. Because mTOR may be associated, at least in part, with mitochondria ***(76,77)***, it is tempting to speculate that Ap4A is another possible signal that connects mitochondrial metabolism to mTOR activity. In this context, it is important to note that Ap4A is a strong inhibitor of AMP-activated protein kinase ***(78)***.

2.3.4. eIF2α

Another mechanism of amino acid sensing is one in which cells respond to changes in the charging of tRNAs. This mechanism is based on data in yeast showing that, upon amino acid starvation, free uncharged tRNA binds

to the protein kinase Gcn2 because its active center strongly resembles that of aminoacyl-tRNA synthetases ***(79)***. Gcn2 activation results in the phosphorylation of eIF2α, which then derepresses GCN4 mRNA translation. Gcn4 is a transcriptional activator that promotes the transcription of many genes involved in nitrogen metabolism; these include not only genes involved in the biosynthesis of amino acids but also genes required for autophagy ***(80)***. The activity of Gcn2 itself becomes inhibited by TOR-dependent phosphorylation of Ser577 of Gcn2 ***(81)***. Because rapamycin is still able to stimulate autophagy in GCN2-disrupted yeast ***(82)***, it is possible that Gcn2 may not be downstream of Tor, or that Gcn2 controls autophagy by a mechanism that is independent of Tor.

In mammalian cells, the eIF2α kinase PKR (double-stranded RNA-dependent protein kinase), which is the equivalent of Gcn2, contributes to the control of autophagy, and PKR can rescue starvation-induced autophagy in GCN2-disrupted yeast ***(82)***.

Unexpectedly, experiments with aminoalcohols, inhibitors of aminoacyl-tRNA synthetases, have yielded contradictory results in mammalian cells with regard to their effects on both amino acid signaling and autophagy, presumably because of lack of specificity of these compounds ***(8,15,59)***. Furthermore, amino acid deprivation did not affect free-tRNA levels, at least in HEK-293 cells ***(43)***.

2.3.5. Phosphatidic Acid

The mitogenic messenger phosphatidic acid can also activate mTOR-dependent signaling provided sufficient amino acids are also present, indicating that phosphatidic acid promotes signaling in parallel to amino acids ***(83,84)***. This is similar to the effect of insulin, as discussed earlier (**Subheading 2.1.**). Whether insulin can affect phosphatidic acid concentrations or whether phosphatidic acid affects autophagy is not known.

2.4. Feedback Interaction in the mTOR Signaling Pathway and Autophagy

Overactivation of amino acid–dependent mTOR-mediated signaling can lead to the inhibition of the proximal part of the insulin signaling pathway ***(85–87)*** (**Fig. 1**). This is because of phosphorylation of IRS1 by S6K, which results in decreased binding of the p85 regulatory subunit of PI3K class I to IRS1. Because class I PI3K is required for mTOR downstream signaling, this feedback system may be part of a homeostatic mechanism that is required to prevent the overactivation of mTOR by amino acids. It has been proposed that the overactivation of mTOR contributes to insulin resistance in obesity-linked diabetics ***(86)***. Downregulation of proximal insulin signaling can be expected to have consequences for autophagy, because a decline in inhibitory

PtdIns(3,4,5)P_3 will accelerate autophagy. Surprisingly, very little is known about protein turnover in type 2 diabetes. It is likely, however, that it is, indeed, increased in type 2 diabetes ***(88,89)***. Whether autophagy contributes to the increased protein turnover in type 2 diabetes is entirely unknown.

The possible consequences of feedback interaction of S6K on mTOR upstream signaling for autophagy were discussed by us ***(90)*** in connection with a study by Scott et al. ***(91)*** concerning the regulation of autophagy in the fat body of *Drosophila melanogaster*. In this study, the authors convincingly show that S6K is not inhibitory, as previously suggested ***(18)*** (*see* **Subheading 2.1.**) but is, in fact, essential for autophagy. Thus, in this insect, the same protein kinase (S6K) that is required for protein synthesis, an anabolic process, is also essential for autophagy, a catabolic process ***(91)***. A possible explanation for this paradox may be found in the negative-feedback effect of S6K on signaling upstream of Tor, which would then increase autophagy as a result of the fall in class I PI3K activity ***(90)***. This may be important, because even under nutrient-rich conditions cells must be able to carry out some autophagic activity, not in order to produce nutrients, but in order to eliminate damaged cell structures or structures that are no longer needed by the cell. Conversely, when nutrients become scarce, e.g., during starvation, the inactivation of Tor by a fall in amino acid concentration accelerates autophagy, provided sufficient active S6K is still present. During long-term starvation, S6K activity may fall so low that class I PI3K is activated again and this would then restrain excessive autophagy in order to prevent cell death, as suggested ***(91)***.

Autophagy has been implicated as a protective mechanism in various neurodegenerative diseases because the process contributes to the removal of defective proteins ***(2)***. In Huntington's disease, for example, expanded polyglutamine proteins accumulate abnormally in intracellular aggregates which in cell models can be prevented by induction of autophagy with rapamycin ***(92)***. Unexpectedly, however, in a functional genetic screen of Huntington's disease it was found that activation of IRS2, which mediates insulin signaling **(Fig. 1)**, results in autophagy-mediated clearance of the polyglutamine protein aggregates ***(93)***. The activation of autophagy occurred in spite of activation of protein kinase B, mTOR, and S6K, but was still PI3K class III- and Beclin1–dependent. Perhaps the negative feedback in proximal insulin signaling by S6K, as discussed in the previous paragraph, accounts for these surprising results.

*2.5. Other Amino Acid–Sensitive Signaling Mechanisms Controlling Autophagy (***see** *Fig. 1)*

In human colon cancer HT-29 cells, another amino acid–dependent signaling pathway can control autophagy, in addition to the PI3K/mTOR pathway.

Activation of Erk1/2 stimulates the GTPase-activating protein GAIP and abolishes the inhibitory effect of trimeric Gi3 protein on autophagy ***(94)***. Amino acids, by stimulating the phosphorylation of Ser^{259}, inactivate the Erk1/2 MAPK kinase Raf-1 and downregulate autophagy ***(9)***. By contrast, in C2C12 myotubes the inhibition of autophagy by amino acids is not accompanied by any changes in Erk1/2 phosphorylation ***(35)***. Differences in amino acid signaling mechanisms and in the control of autophagy may exist, apparently depending on the cell type and perhaps also on the degree of differentiation ***(35)***.

Ceramide has been shown to activate autophagy by upregulation of Beclin 1 and inhibition of protein kinase B ***(95)***. Ceramide also decreases intracellular amino acid concentrations by inhibition of amino acid transport, resulting in decreased mTOR-dependent signaling ***(96)***. Although the link with autophagy was not studied in these studies ***(96)***, inhibition of amino acid transport may also contribute to the activation of autophagy by ceramide.

In cultured rat hepatocytes and in the flow-through perfused rat liver, amino acid–induced cell swelling, caused by Na^+-dependent concentrative transport of certain amino acids, inhibits autophagy independent of mTOR by activation of $p38^{MAPK}$ ***(97)***; likewise, in yeast the $p38^{MAPK}$ orthologue Hog1 also plays an important role in the osmosensitivity of autophagy ***(21)***. Integrins and microtubules are part of the osmo-sensing mechanism ***(10,98)***. Insulin inhibits autophagy because it also increases cell volume and activates $p38^{MAPK}$ by an integrin-dependent, mTOR-independent mechanism ***(38,99)***. By contrast, in C2C12 myotubes, $p38^{MAPK}$ is not involved in the inhibition of autophagy by amino acids ***(35)***, whereas in skeletal muscle and in adipocytes insulin also does not affect $p38^{MAPK}$ ***(100)***.

In contrast to the flow-through perfused rat liver, in the isolated circulatory perfused rat liver, neither amino acids that increase cell volume (glutamine, alanine) nor insulin affect autophagy on their own, unless they are added in combination with other amino acids (e.g., leucine) ***(101)***. In isolated hepatocytes, an increase in cell volume alone is also not sufficient to inhibit autophagy, although it does potentiate the inhibitory effect of low concentrations of leucine and other amino acids on autophagy by a mechanism that is mTOR-dependent ***(18,102)***.

Again, depending on the experimental conditions and on the cell type, different mechanisms appear to operate in the control of autophagy.

3. Concluding Remarks

Although it is now generally accepted that amino acids inhibit autophagy by activation of mTOR-dependent signaling in concert with insulin, very little is known about the mechanisms involved and much work needs to be done

in this exciting area. Among the most compelling questions are the nature of the primary amino acid receptor and the nature of the mechanisms coupling the amino acid signaling pathway to autophagosome formation. If the amino acid receptor turns out to be intracellular rather than extracellular, amino acid metabolism, in addition to plasma membrane amino acid transport, will directly affect intracellular amino acid concentrations, thus signaling and autophagy. Because amino acid metabolism is strongly cell type–dependent, this may also be true for the regulation of amino acid signaling and of autophagy.

Recent reviews on autophagy have stressed the role of autophagy in processes such as cancer and aging ***(103–108)***. An intriguing question here is whether changes in amino acid signaling, in addition to alterations in the expression of Atg proteins, contribute to the variations in autophagy. As discussed earlier (*see* **Subheading 2.4.**), we predict that in type 2 diabetes, which is characterized by insulin resistance, autophagy is activated, possibly as a protective mechanism to eliminate damaged organelles, e.g., mitochondria ***(103)***. In this context it is important to note that both caloric restriction ***(109)*** and defective insulin signaling ***(110,111)*** extend life span; this may be ascribed, at least in part, to increased autophagy ***(109,112)***. It is worthwile mentioning that the Klotho protein, which has strong antiaging properties in mammals, confers insulin resistance ***(113)***. In relation to this, SIRT4, a mammalian homologue of Sir2, which is an NAD-dependent deacetylase that promotes longevity in yeast, flies, and worms, is a mitochondrial enzyme that ADP ribosylates and downregulates glutamate dehydrogenase, inhibits insulin production in ß-cells ***(114)*** (*see* **Subheading 2.3.2**.).

Increased mitochondrial production of reactive oxygen species, with loss of mitochondrial function, has been implicated not only in the aging process ***(109)***, but also in type 2 diabetes ***(115)***; elimination of damaged mitochondria will be of vital importance to prolong life. It is tempting to speculate that diminished amino acid signaling contributes to the autophagic protection under these conditions.

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