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## Addendum

# AMP-Activated Protein Kinase and Autophagy

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## KEY WORDS

mTOR, rapamycin, energy, amino acids, AICAR, metformin, hepatocytes, HeLa cells, HT-29 cells, LKB1

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## Addendum to:

### *AMP-Activated Protein Kinase and the Regulation of Autophagic Proteolysis*

D. Meley, C. Bauvy, J.H.P.M. Houben-Weerts, P.F. Dubbelhuis, M.T.J. Helmond, P. Codogno and A.J. Meijer

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## ABSTRACT

Autophagy is inhibited by TOR-dependent signaling. Interruption of signalling by rapamycin is known to stimulate autophagy, both in mammalian cells and in yeast. However, inactivation of TOR by AMPK has yielded controversial results in the literature with regard to its effect on autophagy: activation of autophagy in yeast but inhibition in hepatocytes. In a recent study, carried out with hepatocytes, HT-29 cells, and HeLa cells, the possible role of AMPK in the control of mammalian autophagy was reexamined. The data suggest that in mammalian cells, as in yeast, AMPK is required for autophagy.

After the original observation in 1995 that amino acids can simultaneously stimulate mTOR-dependent signaling and inhibit autophagy in hepatocytes,<sup>1</sup> it is now generally accepted that the TOR pathway controls autophagy not only in mammalian cells but also in yeast, and interruption of signaling by rapamycin stimulates autophagy.<sup>2</sup> Apart from being a sensor of amino acids, mTOR can also sense changes in the cellular energy state via AMP-activated protein kinase (AMPK) as was simultaneously reported a few years ago by several groups, including our own.<sup>3</sup> Activation of AMPK inhibits mTOR-dependent signaling and inhibits protein synthesis,<sup>3</sup> which is consistent with AMPK's function of switching off ATP-dependent processes.<sup>4</sup>

Inhibition of mTOR by AMPK, like that caused by addition of rapamycin,<sup>2,5</sup> is expected to increase autophagy (Fig. 1). However, the literature on this issue has been controversial. In yeast, activation of AMPK stimulates autophagy.<sup>6</sup> By contrast, activation of AMPK by addition of the cell-permeable nucleotide analogue AICARiboside (AICAR) in hepatocytes strongly inhibits autophagy.<sup>7,8</sup>

Because autophagy is accelerated when cells have insufficient oxidizable substrate at their disposal, inhibition of autophagy by AMPK activation under these conditions was, however, considered to be counterproductive.<sup>5</sup>

Using different mammalian cell types, we have therefore reexamined the possible role of AMPK in the control of autophagy, and the new data<sup>9</sup> indicate that AMPK, like in yeast, is required for autophagy.

The strategy we followed was straightforward and simple. We first repeated, and confirmed, the results obtained by Samari and Seglen<sup>8</sup> with hepatocytes showing that AICAR strongly inhibited flux through the autophagic pathway, measured as 3-methyladenine-sensitive proteolysis. Unexpectedly, activation of AMPK by the anti-diabetic agent metformin appeared to be much less effective in inhibiting autophagy, even though metformin (2 mM) was more potent in activating AMPK in comparison with AICAR at the low concentration of AICAR used in our experiments (250  $\mu$ M). The small residual inhibition of autophagy by metformin could be ascribed to the significant fall (40%) in cellular ATP levels under these conditions, and a large decrease in ATP is known to inhibit autophagy because, after all, autophagy is a complicated membrane-flow-dependent process which does require input of ATP.<sup>10</sup> Subsequently we discovered that, like AICAR which activates AMPK, pharmacological inhibition of AMPK by compound C<sup>11</sup> also inhibited autophagy. An effect of compound C on the lysosomal pH could be ruled out. Inhibition of autophagy by both AICAR and compound C was also observed in HT-29 cells and HeLa cells.<sup>9</sup>

The fact that the AMPK inhibitor compound C strongly inhibited autophagy suggested that AMPK, rather than inhibiting autophagy is in fact required for autophagy, a situation similar to that in yeast.<sup>6</sup> This was supported by experiments with HT-29 cells and HeLa cells showing that transfection of these cells with a gene encoding a dominant negative form of the enzyme (AMPK<sup>DN</sup>) completely inhibited 3-methyladenine-sensitive proteolysis. By contrast, transfection with constitutively active AMPK<sup>CA</sup> did not affect the

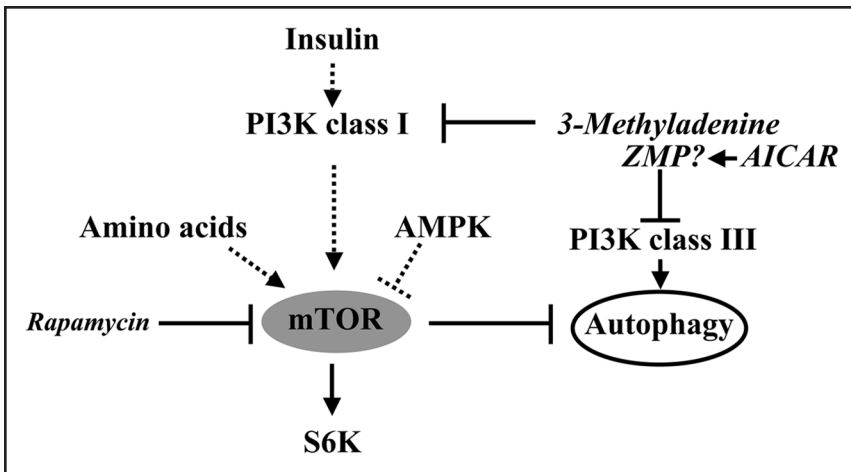


Figure 1. mTOR-dependent signaling and autophagy. Abbreviations: ZMP, the phosphorylated product of AICAR (5-aminoimidazole-4-carboxamide riboside); PI3K, phosphatidylinositol 3-kinase; S6K, p70S6 kinase; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin. The activation of AMPK by ZMP is not indicated in the figure.

rate of autophagy under these conditions. Surprisingly, AMPK<sup>CA</sup> did not activate autophagy in the presence of amino acids; this may be explained by the fact that amino acids can also inhibit autophagy by mTOR-independent mechanisms.<sup>5</sup>

These experiments led us to conclude that AMPK is essential for autophagy and that, apparently, basal activity of AMPK is sufficient for autophagy. Because the AMPK kinase LKB1 is lacking in HeLa cells<sup>12</sup> these data also suggested to us that, apparently, AMPK can also be phosphorylated by another upstream kinase. A possible candidate is calmodulin-dependent protein kinase kinase (CaMKK).<sup>13,14</sup>

We also concluded that the inhibition of autophagy by AICAR is not related to its ability to activate AMPK. There is evidence in the literature that AICAR (or rather ZMP, its phosphorylation product), in analogy with 3-methyladenine, may inhibit phosphatidylinositol 3-kinase (see Ref. 9 and citations therein) and thus interferes with autophagy through inhibition of the class III enzyme which is required for autophagy (see Fig. 1).<sup>2,5</sup> Another spin-off of our studies was the finding that phosphorylation of AICAR to ZMP, and thus its ability to phosphorylate and activate AMPK, was greatly reduced in the presence of amino acids. These observations underscore the warning<sup>15</sup> that variations in the activity of AMPK in the presence of AICAR are not always due to direct effects on AMPK.

The conclusion that AMPK is essential for autophagy is in line with recent data showing that activation of the tumor suppressor p53 inhibits mTOR activity through activation of AMPK, a phenomenon that is accompanied by increased autophagy, of mitochondria in particular.<sup>16</sup> The data are also in agreement with the requirement of autophagy for eukaryotic elongation factor 2-kinase (eEF-2 kinase),<sup>17</sup> which is known to be activated by AMPK.<sup>18</sup>

Interestingly, in many cases where AMPK can be expected to be activated autophagy is known to be increased (although this relation was not considered). For example, autophagy is stimulated in tumor cells when present in a hypoxic environment.<sup>19</sup> Recently, it has been shown that the activation of AMPK by low-oxygen conditions is involved to maintain energy homeostasis in a hypoxic environment.<sup>20</sup> Thus, the activation of AMPK, together with HIF (hypoxic-inducible factor)-dependent signaling that also impinges on the mTOR pathway (reviewed in ref. 21), can contribute to the stimulation of autophagy in tumor cells in a hypoxic environment. Moreover, apoptotic stimuli, which result in increased mitochondrial permeability and decreased mitochondrial membrane potential, target these mitochondria for autophagic degradation.<sup>22,23</sup> Inhibition of mitochondrial ATP synthesis with oligomycin in insect cells was

shown to promote massive autophagy of mitochondria.<sup>24</sup> Likewise, in cerebral ischaemia (mitochondrial) autophagy is triggered.<sup>25</sup>

In this context, the association of mTOR with the mitochondrial outer membrane is noteworthy,<sup>26,27</sup> because a considerable part of cellular adenylate kinase is located in the mitochondrial inter-membrane space. mTOR is therefore ideally located to sense changes in the ATP/AMP ratio<sup>3</sup> and to control autophagy of individual mitochondria.

Although we have not tested this ourselves,<sup>9</sup> these various studies make it tempting to speculate that AMPK may be required for autophagy of mitochondria in particular. In this regard, the localization of AMPK becomes relevant, too. However, the subcellular localization of AMPK is uncertain, but a mitochondrial localization has not been ruled out.<sup>28</sup>

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