

SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

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Our research concerns the role of protein phosphorylation in the control of metabolism by nutrients, hormones and various stresses. As a model system, we studied 6-phosphofructo-2-kinase (PFK-2) /fructose-2,6-bisphosphatase (FBPase-2). This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. Fructose 2,6-bisphosphate was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980 and is the most potent stimulator of 6-phosphofructo-1-kinase (PFK-1), a key enzyme of glycolysis. Fructose 2,6-bisphosphate is synthesised from fructose 6-phosphate and ATP by 6-phosphofructo-2-kinase (PFK-2). Its hydrolysis to fructose 6-phosphate and Pi is catalysed by FBPase-2. These two activities are catalysed at separate sites of a bifunctional enzyme (PFK-2/FBPase-2) composed of two identical subunits.

Our work has focused on the PFK-2 domain, in which we identified the amino acids involved in substrate binding and catalysis. We also proposed a model of the three-dimensional structure of the PFK-2 domain, which was confirmed when the crystal structure became available. We have characterised several PFK-2/FBPase-2 isoforms in mammalian tissues. We also cloned the corresponding mRNAs and showed that they originate from at least two genes (1). These isoforms differ in PFK-2/FBPase-2 activity ratio, kinetic properties and response to phosphorylation by protein kinases. The C-terminus of the heart (H) isozyme, contains phosphorylation sites for several protein kinases. These sites are not present in the other isozymes, such as the liver (L) isozyme, which, by contrast, contains a single phosphorylation site for the cyclic AMP-dependent protein kinase (PKA) at the N-terminus. The concentration of fructose 2,6-bisphosphate changes in response to metabolites, hormones, growth factors, and oncogene activation (1). Over recent years, we made a detailed study of the molecular mechanisms responsible for the activation of heart PFK-2 by insulin and ischemia. This led to the identification of new components of the insulin signalling cascade and to a new interpretation of the Pasteur effect.

Insulin signalling

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SHIP2

SHIP2 (Type II SH2-domain containing inositol 5-phosphatase) antagonises insulin action by hydrolysing phosphatidylinositol 3,4,5-trisphosphate (PIP3) the intracellular second messenger of insulin. We studied mice lacking the SHIP2 gene and found that loss of SHIP2 increased the sensitivity towards insulin, which was characterised by an increased glucose transport and glycogen synthesis in skeletal muscles. This indicates that SHIP2 is a potent negative regulator of insulin signalling and insulin sensitivity (2).

Inhibition of insulin signalling in the ischemic heart

We found that ischemia antagonised insulin signalling in perfused rat hearts. This inhibition resulted from intracellular acidosis, which is a characteristic feature of ischemia. Ischemic acidosis was found to decrease the kinase activity and tyrosine phosphorylation of the insulin receptor, thereby preventing activation of the downstream components of the signalling pathway (3).

Activation of heart PFK-2 by insulin

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate. The mechanism involved in this insulin-induced activation of heart PFK-2 is being studied both *in vitro* and in intact cells. The recombinant heart isozyme of PFK-2 is a substrate of several protein kinases, and especially of protein kinases of the insulin signalling pathways, such as protein kinase B or Akt, which is believed to mediate most metabolic effects of insulin (4). We tested the role of PKB in the activation of PFK-2 by insulin using a 'dominant-negative' construct and found that the activation of PFK-2 by insulin did not require PKB, but was mediated by another protein kinase located downstream of PDK1 (5). We purified a wortmannin-sensitive and insulin-stimulated protein kinase (WISK). WISK phosphorylates heart PFK-2 on Ser466 leading to its activation. Our current efforts are aimed at identifying and cloning this protein

kinase, which differs from known protein kinases of the insulin signalling pathways (6).

AMP-activated protein kinase

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The AMP-activated protein kinase (AMPK) acts as a fuel gauge in the cell. AMPK is activated by an increase in the AMP/ATP ratio as it occurs when the oxygen supply is restricted or after exposure of cells to inhibitors of the mitochondrial respiratory chain, such as oligomycin. In certain cells, AMPK can also be activated by AICA-riboside, which enters cells to be phosphorylated into ZMP, an analogue of AMP. AMPK switches off energy-consuming biosynthetic pathways, thereby conserving ATP.

Stimulation of heart glycolysis by ischemia.

Ischemia or anoxia stimulates glycolysis (Pasteur Effect) which involves increased glucose transport and PFK-2 activation in heart. We investigated whether AMPK could mediate this phenomenon. AMPK phosphorylated heart PFK-2 on Ser 466 which led to its activation. In perfused hearts, ischemia induced an activation of AMPK, which correlated with PFK-2 activation and with an increase in Fru-2,6-P₂ concentration. In cultured HEK-293 cells transfected with heart PFK-2, a dominant negative construct of AMPK abolished both the phosphorylation and activation of transfected PFK-2 induced by oligomycin, an inhibitor of oxidative phosphorylation. Therefore, heart PFK-2 is a new substrate of AMPK and its activation is involved in the Pasteur Effect (7).

Inducible PFK-2

A novel isoform of PFK-2, which is induced by pro-inflammatory stimuli and therefore called inducible PFK-2 (iPFK2), is expressed constitutively in several human cancer lines. In monocytes, iPFK-2 is induced by lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, which triggers an inflammatory response. iPFK-2 resembles the heart isozyme in that it contains

a serine residue (Ser 461) in a similar context to Ser 466 of heart PFK-2 for phosphorylation by AMPK. Recombinant iPFK-2 was indeed phosphorylated and activated by AMPK *in vitro*. In cultured human monocytes activated by LPS, hypoxia activated AMPK and iPFK2. This activation correlated with an increase in the concentration of fructose 2,6-bisphosphate and with a stimulation of the glycolytic flux. In

cultured HEK-293 cells, a dominant-negative construct of AMPK abolished the activation of transfected iPFK2 by oligomycin. We propose that the stimulation of monocyte glycolysis via the AMPK-induced phosphorylation and activation of iPFK-2 could be important for furnishing ATP to sustain cytokine synthesis in infected anaerobic tissues (8).

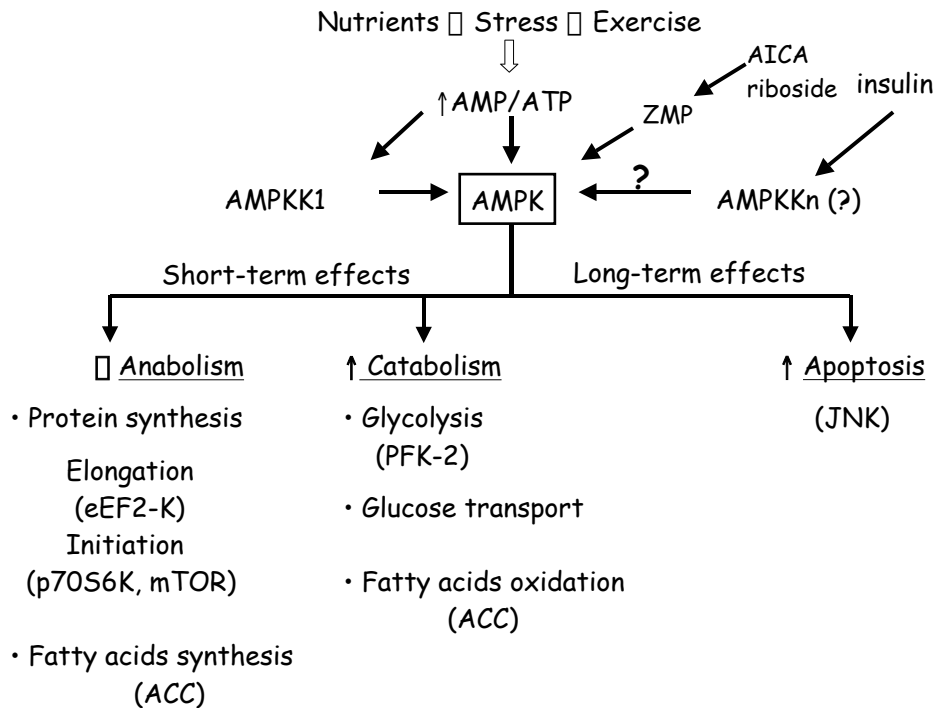


Fig. 1. Mechanism of activation and targets of AMP-activated protein kinase (AMPK). AMPK is phosphorylated and activated by AMPKK when the AMP/ATP ratio increases as a result of metabolic stresses. It can also be activated by an AMP analogue, ZMP, which is formed from AICA-ribose. Insulin antagonises AMPK activation by a still unknown mechanism. The targets of AMPK, which we discovered and which are responsible for some of its short-and long-term effects, are indicated. AMP, adenosine monophosphate; ATP, adenosine triphosphate; AICA riboside, 5-aminoimidazole-4-carboxamide riboside; ZMP, AICA ribotide; AMPK, AMP-activated protein kinase, AMPKK, AMPK-kinase; eEF2K, eukaryotic elongation factor-2-kinase; p70S6K, p70 ribosomal protein S6 kinase; mTOR, mammalian target of rapamycin; ACC, acetyl-CoA carboxylase; PFK-2, 6-phosphofructo-2-kinase; JNK, c-jun kinase

AMPK activation inhibits protein synthesis

Protein synthesis, in particular peptide chain elongation, consumes a large proportion of intracellular ATP. Therefore, we investigated whether AMPK activation could inhibit protein synthesis via the phosphorylation of regulatory components of the translation machinery. In anoxic rat hepatocytes or in hepatocytes treated with 5-aminoimidazole-4-carboxamide (AICA) riboside, AMPK was activated and protein synthesis was inhibited. The inhibition of protein synthesis could not be explained by changes in the phosphorylation states of factors known to control the initiation of translation, such as factor 4E-binding protein-1 or eukaryotic initiation factor 2a. However, the phosphorylation state of eukaryotic elongation factor 2 (eEF2) was increased in anoxic hepatocytes. In HEK-293 cells, transfection of a dominant negative AMPK construct abolished the oligomycin-induced inhibition of protein synthesis and eEF2 phosphorylation. Lastly, eEF2 kinase, the kinase that phosphorylates eEF2, was activated in anoxic or AICA riboside-treated hepatocytes. Therefore, the activation of eEF2 kinase by AMPK, resulting in the phosphorylation and inactivation of eEF2, provides a novel mechanism for the inhibition of protein synthesis (9).

Incubation of hepatocytes with amino acids, such as glutamine and leucine, leads to an activation of biosynthetic pathways, such as glycogen synthesis, lipogenesis and protein synthesis. Under these conditions, p70 ribosomal S6 kinase (p70S6K), a protein kinase that participates in the control of protein synthesis and is activated in response to hormones, mitogens and nutrients, becomes activated via activation of the mammalian target of rapamycin (mTOR) by an unknown mechanism. Pretreatment of hepatocytes with AICA riboside prevented the activation and phosphorylation of p70S6K. Therefore, it is likely that AMPK inhibits p70S6K activation by phosphorylating target(s) in the mTOR signalling pathway.

Sustained activation of AMPK triggers apoptosis in liver cells

We studied the effect of long-term AMPK activation on liver cell survival. AMPK activation was maintained in FTO2B cells treated with AICA riboside or by adenoviral transfection of hepatocytes with constitutively active AMPK. Sustained AMPK activation triggered apoptosis through an activation pathway involving c-Jun kinase and caspase-3 (10).

AMPK activation by upstream kinases

AMPK activation requires phosphorylation in the activation loop at Thr172 of its catalytic alpha-subunit

by an upstream kinase, AMPK-kinase, itself sensitive to AMP. We studied the effect of insulin on this phenomenon. We observed that AMPK activation in hypoxic hearts was antagonised by a pre-treatment of the hearts with insulin. The effect of insulin was blocked by wortmannin, an inhibitor of PI 3-kinase and resulted in a decreased phosphorylation state of Thr172 in AMPK. In addition, the insulin effect was unrelated to changes in the AMP/ATP ratio, thus demonstrating that AMPK activity could be modified by a mechanism independent of the AMP/ATP ratio in cardiomyocytes.

As stated above, evidence is growing that other kinases (AMPKKs), which are not AMP-sensitive, are also involved in the control of AMPK activity. Using partially purified AMPKK to phosphorylate bacterially expressed AMPK heterotrimers, we have identified upstream kinase phosphorylation sites in AMPK by mass spectrometry (see below). In addition to confirming the phosphorylation of Thr 172, new sites have been identified in the α -1 and α -2 catalytic subunits. The role of these sites is being studied by site-directed mutagenesis and transfection. The AMPKKs are being purified from heart and liver for characterisation.

Stimulation of heart glycolysis by increased work

In skeletal muscle, contraction activates AMPK and stimulates glycolysis by promoting glucose transport. Therefore, we investigated whether submitting hearts to an increased workload would stimulate glycolysis via the AMPK-induced activation of PFK-2. Increasing the workload indeed activated PFK-2, but AMPK activity was unchanged. The PI 3-kinase inhibitor, wortmannin, counteracted the workload-induced stimulation of glycolysis and PFK-2 activation. Moreover, PKB was activated by increasing the workload and this effect was abrogated by wortmannin. We conclude that increasing heart work stimulates glycolysis through the PI 3-kinase/PKB or WISK signalling pathway independently of AMPK. This contrasts with the situation in contracting skeletal muscle and ischemic heart, where the stimulation of glycolysis involves AMPK activation.

Control of smooth muscle contraction

D. Vertommen and M.H. Rider in collaboration with P. Gailly, UCL, D. Carling, London, and M. Walsh, Calgary, Canada

In earlier work, we used nanoelectrospray- and on-line capillary- electrospray ionisation mass spectrometry (ESI-MS) to identify

autophosphorylation and regulatory phosphorylation sites in the novel protein kinase, protein kinase D (PKD). However, there are no physiological substrates yet recognised for this protein kinase. We have shown that in various smooth muscles stimulated with vasoconstrictors, such as vasopressin, PKD becomes activated. Moreover, we have discovered new *in vitro* substrates for PKD in the contractile machinery that could be implicated in the prolonged phase of muscle contraction when Ca²⁺-dependent P-light chain phosphorylation of myosin is not involved in force generation. We are currently looking at the agonist-induced development of force in femoral artery strips to see whether, using mass spectrometry, the phosphorylation sites on proteins phosphorylated by PKD *in vitro* are phosphorylated in response to agonists *in vivo*. This work has potential for the understanding of hypertension and could lead to new treatments for this condition.

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