Glucose-dependent regulation of AMP-activated protein kinase in MIN6 beta cells is not affected by the protein kinase A pathway

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1. Introduction

Mammalian AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of the cellular energy status. AMPK is a heterotrimer composed of the catalytic AMPK\(a\) and two regulatory subunits, one involved in the binding of AMP, ADP and ATP (AMPK\(\gamma\)) and the other acting as a scaffold that determines the subcellular localization of the complex (AMPK\(\beta\)) [1]. Several isoforms of each subunit have been described (\(\alpha\), \(\beta\), \(\gamma\), \(\gamma\), \(\gamma\), \(\gamma\), \(\gamma\), \(\gamma\)) that are expressed in a tissue-specific manner. AMPK activity is regulated by two mechanisms: (i) allosteric activation by AMP, and (ii) phosphorylation of Thr172 in the catalytic domain of AMPK\(a\) by the upstream kinases LKB1 and CaMKK\(\beta\) [1]. It has recently been proposed that upon binding of AMP or ADP to the AMPK\(\gamma\) subunit, the AMPK complex undergoes a conformational change that makes it a poorer substrate for dephosphorylation [2,3]. Therefore, an AMP/ADP-induced conformational switch and the ensuing phosphorylation of the catalytic subunit on Thr172 are the key factors that determine the activity of AMPK.

AMP-activated protein kinase (AMPK) is a sensor of cellular energy status. In pancreatic beta cells, glucose induces the dephosphorylation of Thr172 within the catalytic subunit and the inactivation of the AMPK complex. Here we demonstrate that glucose also activates protein kinase A (PKA), leading to the phosphorylation of AMPK\(a\) at Ser485 and Ser497. However, these modifications do not impair the phosphorylation of Thr172 by upstream kinases, and phosphorylation of Thr172 does not affect the phosphorylation of AMPK\(a\) by PKA either. Thus, although phosphorylation of Thr172 and Ser485/Ser497 are inversely correlated in response to glucose, they follow an independent regulation.

In pancreatic beta cells AMPK activation impairs glucose-stimulated insulin secretion [4,5]. This effect raises some concerns about the use of general activators of AMPK in the treatment of different pathologies [6]. To overcome the action of AMPK on glucose-stimulated insulin secretion, glucose induces the rapid dephosphorylation and inactivation of the pancreatic AMPK complex [4,7]. In fact, we have recently demonstrated that the PP1-R6 protein phosphatase holoenzyme, composed of protein Ser/Thr phosphatase PP1 and the R6 (PPP1R3D) regulatory subunit, participates in the glucose-induced dephosphorylation and inactivation of AMPK [8]. In this way, glucose inactivates AMPK and, at the same time, induces insulin production and secretion in pancreatic beta cells.

Recently, an alternative mechanism for the downregulation of AMPK activity has been described. In adipocytes, protein kinase A (PKA) phosphorylates AMPK\(a\) on Ser173, Ser485 and Ser497, and this impedes the phosphorylation of Thr172 by upstream activating kinases [9]. These results are consistent with a previous report indicating that in the INS1 beta cell line, activation of the PKA pathway in response to drugs that elevate cellular cAMP results in an enhancement of the phosphorylation of AMPK\(a\) at Ser485 which leads to the reduction of the phosphorylation status of Thr172 and the inhibition of AMPK activity [10]. However, there are conflicting results on the negative role of PKA on the regulation of AMPK activity: it was reported that in human umbilical vein
endothelial cells, treatment with forskolin, a drug which activates the PKA pathway, increased the phosphorylation of AMPK at both Thr172 and Ser485 sites [11], and that in adipocytes, activation of PKA by isoproterenol or forskolin also increased phosphorylation of Thr172 and AMPK activity [12]. Another protein kinase has been implicated in the regulation of AMPK activity. In heart preparations, protein kinase B (PKB) also phosphorylates AMPKα at Ser485, and this prevents LKB1 phosphorylation at Thr172, decreasing the activity of AMPK [13]. However, it was not clear at that time whether PKB could phosphorylate AMPKα at alternative sites. A more direct analysis of the effect of the modification of the Ser485 site on AMPK activity was obtained when AMPKα Ser485Aα and Ser485Asp mutants were analyzed. It was reported that none of these mutants affected the overall AMPK activity, so it was proposed that phosphorylation of this residue did not participate directly in the regulation of AMPK activity [14].

To explore whether the glucose-dependent phosphorylation of AMPK (either by PKA or PKB) plays a role in the glucose-dependent inhibition of AMPK in beta cells, we checked the phosphorylation status of AMPKα1 at Ser485 and Ser497 in response to glucose and assessed whether there was any correlation between the phosphorylation of AMPK at these sites and the phosphorylation status of Thr172, an indicator of AMPK activation. Here, we present evidence indicating that, although glucose regulation of the phosphorylation status of AMPKα1 at Thr172 and Ser497/Ser485 by upstream kinases and PKA/PKB, respectively, are inversely correlated, the phosphorylation of these sites follows an independent regulation.

2. Materials and methods

2.1. Cell culture

Mouse insulinoma pancreatic beta cells (MIN6) were kindly provided by Dr. Jun-Ichi Miyazaki (Osaka University, Japan). Cells (passage 20–30) were grown and handled as described in [8]. Forskolin, glyburide and nifedipine were obtained from Sigma (Madrid, Spain). Plasmids pcDNA-AMPKα1, pcDNA-AMPKα1S485A, pcDNA-AMPKα1S485D, pcDNA-AMPKα1S497A and pcDNA-AMPKα1S497D have been previously described [9]. Plasmid pcDNA-AMPKα1S485A/S497A was obtained by subcloning a fragment from plasmid pHIS-AMPKα1S485A/S497A-J1-γ1 into pcDNA3.

2.2. Small interference RNA silencing

Cells were treated with a negative control siRNA (mock) or with siRNA oligonucleotides for PP1α and PP1β (Ambion, Madrid, Spain) as in [8].

2.3. Immunoblotting

Cell lysis and protein extracts were obtained and analyzed as in [8]. Cell lysis (30 μg) were boiled in electrophoresis sample buffer and analyzed by SDS/PAGE and immunoblotting using appropriate antibodies: anti-AMPKα, anti-phospho-AMPKα-Thr172, anti-phospho-AMPKαSer485, anti-AMPKβ1, anti-phospho-LKB1-Ser428 and anti-phospho-ACC-Ser79, from Cell Signaling Technology (Hertfordshire, UK); anti-PP1α (C-19) from Santa Cruz Biotechnology (Santa Cruz, USA); anti-PP1β from Sigma (Madrid, Spain); anti-phospho-CREB-Ser133 from Millpore (Temecula, USA); and anti-phospho-AMPKαSer497 [9]. Secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Immunoblots were analyzed with the ECL + reagent (GE Healthcare, Barcelona, Spain) and chemiluminescence was detected using a FUJIFILM LAS-3000 lite imager.

3. Results

3.1. Glucose induces phosphorylation of AMPKα1 at residues Ser485 and Ser497 and also dephosphorylation of Thr172

Glucose is a negative regulator of the AMPK activity in different mammalian cell models, including pancreatic beta-cells ([4,7]). Since activation of cAMP-dependent protein kinase A (PKA) by either lipolytic hormones in adipocytes [9] or cAMP elevating drugs in INS1 beta cells [10] prevents the activating phosphorylation of AMPKα1 at Thr172, we decided to study whether PKA is also implicated in the glucose-induced inhibition of AMPKα in pancreatic beta-cells. With this aim, we used mouse pancreatic MIN6 beta-cells, a model for glucose-responsive primary beta-cells [15], which mainly expresses AMPK of the α1β1γ1 type [7]. MIN6 cells were grown under conditions of high (25 mM) or low (3 mM) glucose before the determination of the phosphorylation status of endogenous AMPKα1 on Thr172 (as a sign of AMPK activation) and Ser485 and Ser497 (as indicators of PKA phosphorylation; we assume that phosphorylation of Ser173 also happens at the same time, but as we do not have good antibodies to detect endogenous levels of this phosphosite, we do not present results on this modification), using phospho-epitope specific antibodies. As shown in Fig. 1 (lane a), in cells growing in low glucose (3 mM) AMPKα1 was heavily phosphorylated on Thr172 but only marginally on Ser497. However, if cells were exposed to a high glucose concentration (25 mM) for 1 h, the phosphorylation of Thr172 was reduced, in agreement with previous reports ([7,8]), whereas the phosphorylation of Ser485 and Ser497 was clearly increased (Fig. 1, lane b). Thus, glucose induced both the dephosphorylation of Thr172 and the phosphorylation of Ser485 and Ser497. Intriguingly, the phosphorylation of AMPKα1 at Thr172 and Ser485/Ser497 follows an inverse correlation: if cells grown in low glucose were shifted to high glucose for 1 h and then shifted back to low glucose for an extra hour, the phosphorylation levels of Thr172 and Ser485/Ser497 were reversed again (Fig. 1, lane c). Likewise, if cells were grown overnight in high glucose and then shifted to low glucose conditions, the phosphorylation of Thr172 and Ser485/Ser497 increased and decreased, respectively (Fig. 1, lanes d and e). In agreement with published observations, the phosphorylation status of Thr172 correlated with an increase in AMPK activity, assessed by the phosphorylation status of one its targets, namely acetyl-CoA carboxylase (ACC) at Ser79 (Fig. 1).

3.2. Phosphorylation of AMPKα1 by PKA does not prevent phosphorylation of Thr172 by upstream kinases

It is known that glucose activates PKA in pancreatic beta-cells [16]. To delineate a putative role of PKA in the glucose-induced inhibition of AMPKα phosphorylation at Thr172, cells growing in high glucose were shifted to low glucose conditions in the presence or absence of 10 μM forskolin, a potent activator of adenylate cyclase and PKA signaling. We confirmed that forskolin activated PKA signaling by measuring the phosphorylation status of the transcription factor CREB (pSer133-CREB), a well-established nuclear substrate of PKA (Fig. 2A, lanes d and e). It should be pointed out that CREB is only transiently phosphorylated by PKA [17], which also explains why this phosphorylation is not observed in cells growing overnight in high glucose. Forskolin also promoted the phosphorylation of LKB1 (pSer428-LKB1), in agreement with the described PKA-dependent phosphorylation of LKB1 [18] (Fig. 2A, lanes d and e), and the phosphorylation of AMPKα1 at residues Ser485 and Ser497 (Fig. 2A, lanes d and e). However, we did not observe changes in the rate of phosphorylation of Thr172 induced by a shift from high to low glucose conditions, in cells treated or not
with forskolin (Fig. 2A), indicating that the phosphorylation of AMPK by PKA did not affect the phosphorylation of Thr172 by upstream kinases.

We also analyzed whether the absence of an active PKA pathway affects the phosphorylation of AMPKα1 at Thr172. For that purpose we treated cells with specific siRNAs to deplete the expression of the major PKA isoforms (PKAα1 and PKAβ1) (Supplementary Fig. S1). Although this treatment efficiently diminished the activity of PKA, as illustrated by the decreased phosphorylation of pSer133-CREB, it did not affect the corresponding dephosphorylation of Thr172. As expected, the glucose-induced phosphorylation of Ser497 was reduced, albeit only partially. Therefore, the absence of an active PKA pathway does neither interfere with the glucose-induced phosphorylation of Ser497, nor the glucose-induced dephosphorylation of AMPKα1 at Thr172 nor with the phosphorylation of this residue by upstream kinases under low glucose conditions.

In order to clarify the role of the phosphorylation of residues Ser485 and Ser497 on the regulation of the phosphorylation of Thr172 by glucose, we transfected MIN6 cells with plasmids expressing wild type AMPKα1, or mutants in which the phosphorylation of these sites was prevented (S485A, S497A or the double S485A/S497A), or the phosphomimetic S485D or S497D mutants. As shown in Fig. 2B, and in agreement with previous reports [14], regulation of the phosphorylation of Thr172 by glucose followed a similar trend in any of the assayed mutated forms, without any significant difference in the levels of this phosphosite among them. Therefore, the phosphorylation of residues Ser485 and Ser497 does not affect the regulation of the phosphorylation of Thr172 by glucose. Our results suggest that the regulation of pThr172 by glucose only happened on the pool of AMPKα1 subunits forming part of the trimeric AMPK complex (similar levels of pThr172 were present in cells overexpressing or not AMPKα1), but not on free AMPKα1 subunits, present in excess. These results also indicate that the phosphorylation status of Ser485 or S497 in free AMPKα1 subunits was constitutive. Moreover, the phosphorylation of these residues was affected by the presence of a non-phosphorylatable residue in the other site. Perhaps the introduction of a non-phosphorylatable residue in one of these sites prevents the access of the other site to its corresponding kinase.

3.3. Phosphorylation of AMPKα1 at Thr172 does not interfere with the phosphorylation of residues Ser485 and Ser497

We also analyzed whether phosphorylation of AMPKα1 at Thr172 affects the phosphorylation of Ser485 and Ser497. We have recently demonstrated that the glucose-induced dephosphorylation of AMPKα1 at Thr172 is prevented by depleting MIN6 cells of the PPP1CA and PPP1CB transcripts, encoding the PP1α and PP1β isoforms of protein phosphatase PP1, respectively [8]. Hence, we studied the glucose-induced phosphorylation of Ser485 and Ser497 before and after depletion of these two major PP1 isoforms. As shown in Fig. 3A, depletion of PP1α and PP1β, as confirmed by immunoblotting, partially prevented the glucose-induced dephosphorylation of AMPKα1 at Thr172 (Fig. 3, compare lanes b and d), in accordance with previous data [8]. However, this depletion did not impair the glucose-induced phosphorylation of residues Ser485 and Ser497, or the glucose induced phosphorylation of LKB1 at Ser428 (a sign of PKA activation). These results suggested that the phosphorylation status of Thr172 does not interfere with the phosphorylation of Ser485 and Ser497.

As an independent approach to further explore this notion, we treated cells with glyburide, an inhibitor of K_{ATP}-sensitive channels. The closure of these channels leads to a depolarization of the plasma membrane and the opening of L-type voltage-dependent
Ca\textsuperscript{2+} channels (VDCC). The resulting increase in the intracellular Ca\textsuperscript{2+} levels activates a Ca\textsuperscript{2+}-dependent adenylate cyclase isoform (AC type 3) in pancreatic beta-cells, the production of cAMP and the activation of PKA (see\cite{19} for review). Therefore, treatment of the cells with glyburide activates PKA independently of glucose metabolism. As expected, in glyburide treated cells grown under low glucose conditions, the phosphorylation status of Thr172 was not affected (Fig. 3B). However, we observed a clear activation of PKA, assessed by an increase in the levels of pSer133-CREB, and a clear increase in the phosphorylation of Ser485 and Ser497 in AMPK\alpha. Transfected cells were grown overnight in high glucose medium (25 mM) and then shifted to low glucose medium (3 mM) for 1 h. Cell extracts were prepared as in Fig. 1 and analyzed using phospho-AMPK\alpha T172, -S485, -S497 and anti-AMPK\alpha antibodies. Higher levels of total AMPK\alpha were detected in cells transfected with plasmids containing different AMPK\alpha forms. A higher exposure of the blot corresponding to the anti-phospho-S497 antibody is shown to demonstrate that the mutant overexpressed forms are not recognized by this antibody. Similar results were obtained with the anti-phospho-S485 antibody (not shown).

3.4. The blockage of the entry of Ca\textsuperscript{2+} into pancreatic beta-cell does not affect the dephosphorylation of Thr172 induced by glucose

It has recently been reported that an intracellular Ca\textsuperscript{2+} rise is needed to promote the glucose-induced dephosphorylation of eIF2\alpha by PP1 in beta cells\cite{20}. In order to check whether Ca\textsuperscript{2+} entry is also necessary for the glucose-induced dephosphorylation of AMPK\alpha1 at Thr172, we treated cells with nifedipine, an inhibitor of L-type Ca\textsuperscript{2+} channels, avoiding in this way the entry of Ca\textsuperscript{2+} via the plasma membrane\cite{16}. Cells growing in low glucose conditions were pretreated or not with 50 \textmu M nifedipine and then shifted to high glucose conditions. As expected, nifedipine treatment blocked Ca\textsuperscript{2+} entry and decreased the activity of PKA pathway, as indicated by the lower levels of pSer133-CREB (Fig. 4). The glucose-induced phosphorylation of AMPK\alpha1 at Ser485 and Ser497 was also reduced by a decreased PKA activity. However, the glucose-induced...
dephosphorylation of Thr172 followed a similar trend in nifedipine treated or untreated cells (Fig. 4). Therefore, the blockage of the entry of Ca$^{2+}$ does not affect the glucose-induced dephosphorylation of Thr172.

4. Discussion

A tight regulation of the AMP-activated protein kinase (AMPK) complex is of paramount importance in view of its key functions in cell metabolism. Cumulative evidence indicates that the activation of AMPK depends on the phosphorylation status of Thr172 of the catalytic subunit. This phosphorylation results from the action of upstream kinases (which promote activation) and the action of specific phosphatases (which promote inactivation). Here, we report that in MIN6 pancreatic beta cells, glucose triggers both the dephosphorylation of Thr172 and the phosphorylation of residues Ser485 and Ser497 in AMPK$\alpha_1$, leading to the inactivation of the AMPK complex. Although we have observed that the phosphorylation of AMPK$\alpha_1$ at Thr172 does not interfere with the phosphorylation of residues Ser485 and Ser497 by PKA.
tion status of Thr172 and Ser485/Ser497 residues is inversely correlated, our results suggest that the regulation of the phosphorylation of these sites is independent of each other. In addition, our results indicate that the phosphorylation of Ser485 and Ser497 do not affect the phosphorylation status of Thr172, and probably the activity of AMPK. Apparently, these results are in conflict with previous reports which indicated that in INS1 beta cells, activation of the PKA pathway in response to drugs that elevate cellular cAMP leads to the reduction of the phosphorylation status of Thr172 [10].

Perhaps the different cell line used could explain the discrepancy of the results. It has also been described that protein kinase B (PKB) also phosphorylates AMPKα at Ser485, and this prevents LKB1 phosphorylation at Thr172, decreasing the activity of AMPK [13]. Perhaps the use of different cell types (heart muscle cells versus MIN6 cells) or the phosphorylation of AMPKα by PKB at alternative sites, could account for these paradoxal findings.

We also report that the regulation of the phosphorylation status of Thr172 is not affected by the closure of the K ATP-sensitive channel (KATP).

Fig. 4. The glucose-induced entry of Ca2+ inside pancreatic beta-cell does not affect the dephosphorylation of Thr172 induced by glucose. MIN6 cells were grown overnight in low glucose medium (3 mM) and then pretreated or not with 50 μM nifedipine (an inhibitor of L-type Ca2+ channels) for 30 min. Then cells were shifted to high glucose medium containing or not 50 μM nifedipine for the indicated times. Cell extracts were prepared as described in Fig. 1 and analyzed using the indicated phosphospecific antibodies. Extracts were also analyzed with anti-AMPKβ1 as loading control. On the right, the intensity of the bands related to the levels of AMPKβ1 is plotted (values are means ± S.E.M. of three independent experiments (‘P < 0.05, ‘‘P < 0.01) analyzed by independent sample ‘t’ test.

Fig. 5. Proposed model of independent regulation of the phosphorylation status of Thr172, Ser485 and Ser497. See text for details. KD, kinase domain in AMPKα; AMP sites in AMPKγ subunit filled with AMP (black oval) or with ATP (white rectangle).
channels (which triggers the entry of Ca\(^{2+}\)) or by the blockage of the entry of Ca\(^{2+}\) inside the cells. Although it cannot be discarded that other glucose triggered signals may affect the phosphorylation of Thr172, we favor the hypothesis that glucose mainly regulates the dephosphorylation of Thr172 by altering the levels of ATP and by enhancing the action of a specific phosphatase involved in the dephosphorylation of this residue (PP1-R6). Thus, we propose the following order of events in the (in)activation of AMPK (Fig. 5): when the glucose concentration is low, AMPK\(\alpha\)1 is active and fully phosphorylated at Thr172. In these conditions AMP levels are high, which keeps AMPK\(\gamma\) in a conformational state that prevents the dephosphorylation of Thr172 by the protein phosphatase PP1-R6, which is bound to the AMPK\(\beta\) subunit [8]. When the glucose concentration increases, AMP levels drop and AMPK converts to a conformation that is accessible for dephosphorylation of Thr172 by protein phosphatase PP1-R6. Independently, glucose also triggers the production of CAMP, leading to the activation of PKA and the phosphorylation of AMPK\(\alpha\)1, at least on residues Ser485 and Ser497. If glucose becomes scarce, the increase in AMP/ADP levels would induce a change in the conformation of the AMPK complex, which would become a poorer substrate for phosphatases, and LKB1, the main AMPK upstream kinase in beta cells, will gain access to phosphorylate Thr172 resulting in AMPK activation. At the same time, Ser485/Ser497 residues will become dephosphorylated by an hitherto unidentified phosphatase (PKA-PPase). Since LKB1 appears to be constitutively active, our results point to a crucial role of the PP1-R6 phosphatase holoenzyme in the regulation of the activity of AMPK in MIN6 cells.

Acknowledgments

We want to thank Dr. Jun-Ichi Miyazaki (Osaka University, Japan) for the MIN6 beta cells. This work was supported by a grant from the Spanish Ministry of Education and Science (SAF2011-27442) and a grant from Generalitat Valenciana (Prometo 2009/051).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.10.032.

References