Inhibition of the mTOR/p70S6K pathway is not involved in the insulin-sensitizing effect of AMPK on cardiac glucose uptake

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Ginion A, Auquier J, Benton CR, Mouton C, Vanoverschelde J-L, Hue L, Horman S, Beauloye C, Bertrand L. Inhibition of the mTOR/p70S6K pathway is not involved in the insulin-sensitizing effect of AMPK on cardiac glucose uptake. Am J Physiol Heart Circ Physiol 301: H469–H477, 2011. First published May 20, 2011; doi:10.1152/ajpheart.00986.2010.—The AMP-activated protein kinase (AMPK) is known to increase cardiac insulin sensitivity on glucose uptake. AMPK also inhibits the mammalian target of rapamycin (mTOR/p70 ribosomal S6 kinase (p70S6K) pathway. Once activated by insulin, mTOR/p70S6K phosphorylates insulin receptor substrate-1 (IRS-1) on serine residues, resulting in its inhibition and reduction of insulin signaling. AMPK was postulated to act on insulin by inhibiting this mTOR/p70S6K-mediated negative feedback loop. In addition, this resistance is linked, in part, to the impairment of the translocation of the glucose transporter GLUT4 to the plasma membrane (see Ref. 10 for a review). The defect in GLUT4 translocation is, moreover, associated with an alteration of the insulin-induced activation of the protein kinase B (PKB)/Akt signaling pathway (17, 26).

In nondiabetic cardiomyocytes, insulin binding to its receptor induces the recruitment and the phosphorylation on tyrosine residues of the insulin receptor substrate-1 (IRS-1; see ref. 5 for a review). This leads to the activation of the phosphatidylinositol 3-kinase, which in turn allows the corecruitment of the phosphoinositide-dependent kinase-1 (PDK1) and PKB/Akt. Once recruited, PDK1 phosphorylates and activates PKB/Akt. One of the substrates of PKB/Akt is the Akt substrate 160 (AS160), the GTPase-activating protein (GAP) of the small G-protein Rab family required for the regulation of the trafficking of GLUT4 vesicles. In the basal state, AS160 inhibits GLUT4 translocation. The PKB/Akt-dependent phosphorylation of AS160 prevents its Rab GAP function and, so, favors GLUT4 translocation to the plasma membrane. The decrease of insulin signaling found in insulin-resistant tissues can be linked, among others, to the phosphorylation of IRS-1 on serine/threonine residues inducing its inactivation (9). Phosphorylation of IRS-1 on Ser636/639 by the mammalian target of rapamycin (mTOR/p70 ribosomal S6 kinase (p70S6K) pathway is of particular interest. Indeed, mTOR/p70S6K pathway is itself a downstream target of PKB/Akt (5). This insulin-induced activation of mTOR/p70S6K provokes the phosphorylation and inactivation of IRS-1 in a self-attenuated mechanism by which insulin negatively regulates its own signaling upon prolonged stimulation (33, 36). It is tempting to postulate that the inhibition of this negative feedback loop should increase insulin sensitivity (9). This hypothesis can be linked to the AMP-activated protein kinase (AMPK). Considered as a cellular fuel gauge, AMPK is activated during cardiac ischemia (see Refs. 11, 38, 41 for reviews). More recently, it has been shown that AMPK can be activated by the insulin-sensitizing and antidiabetic drug metformin (12, 15, 43). AMPK, independently of insulin, is able to phosphorylate AS160 (19, 27) and to stimulate glucose uptake (4, 29) in the heart. In addition, AMPK inhibits the mTOR/p70S6K pathway first discovered to be involved in the regulation of protein synthesis (5). Taking into account the negative effect of AMPK on mTOR/p70S6K, it is speculated that AMPK activation, by inactivating the mTOR/p70S6K-mediated negative feedback loop, should prevent IRS-1 inhibition and should, then, increase insulin sensi-
tivity (14). In line with this hypothesis, we previously showed that activation of AMPK by various activators, including metformin, phenformin, and the non-antidiabetic drug oligomycin, increased insulin signaling (monitored by PKB/Akt phosphorylation state measurement) and overstimulated insulin-induced glucose uptake in both insulin-sensitive and -resistant primary cultured cardiomyocytes (4). The aim of the present work was to prolong this initial study by 1) investigating the interplay of insulin and AMPK activators on different elements of the insulin signaling pathway including IRS-1, p70S6K, and AS160; and 2) evaluating the role of the inhibition of the insulin-induced negative feedback loop in the insulin-sensitizing effect of AMPK activators by using rapamycin the specific inhibitor of the mTOR/p70S6K pathway.

**MATERIALS AND METHODS**

Unless otherwise stated, the source of all materials is given in Refs. 4, 42.

**Ethical information.** This study was approved by the Animal Research Committee of the Université Catholique de Louvain.

**Preparation and treatment of adult rat cardiomyocytes in primary culture.** Cultured adult cardiomyocytes were prepared from male Wistar rats as described previously (4). The cardiomyocytes were equally distributed in laminin-coated dishes and incubated at 37°C for 1 h in medium 199 (Invitrogen; 5.5 mM glucose), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultured dishes were then washed with PBS to remove nonattached death cells. Dishes were then randomly assigned to receive fresh medium 199 [supplemented with 2 mM carnitine, 5 mM creatine, 5 mM taurine, 10−10 M T3, 0.2% (w/v) BSA (fatty acid free), 100 U/ml penicillin, and 100 μg/ml streptomycin] in the presence of 10−8 M insulin to maintain insulin sensitivity or 10−7 M insulin to induce insulin resistance. After 24 h, the dishes were then washed several times with PBS, reincubated for 1 h in minimal medium 199 without insulin, and treated as described in RESULTS. After treatment, the medium was removed and kept for the measurement of glucose uptake. The cells were then lysed in a buffer containing 50 mM HEPES (pH 7.5), 50 mM KF, 1 mM KPi, 5 mM EDTA, 5 mM EGTA, 15 mM β-mercaptoethanol, a standard protease inhibitor mixture (Complete Mini; Roche), and 0.2% (vol/vol) Triton. The lysates were then centrifuged (10,000 g, 15 min, 4°C), and the supernatants were stored at −80°C.

**Glucose uptake measurement.** In muscle, glucose uptake (i.e., glucose transport and phosphorylation) approximates to the rate of glucose transport, because the rate of phosphorylation by hexokinase exceeds that of glucose transport. We measured glucose uptake by the detritiation rate of [2-3H]glucose (6), which occurs after glucose phosphorylation during the rapid isomerization of hexose-6-phosphates catalyzed by phosphoglucose isomerase. Because the rate of this reaction is ~100 times faster than that of hexokinase, the rate of detritiation adequately estimates glucose uptake as defined above. To measure glucose detritiation, tracer amounts (0.2 μCi/ml; PerkinElmer) of tritiated glucose were added to the medium (including 5.5 mM glucose) 30 min before the end of the different treatments. Medium was then taken to separate tritiated water from tritiated glucose by column chromatography (6).

**Phosphorylation state measurements.** The phosphorylation state of AMPK, PKB/Akt, p70S6K, IR, and IRS-1 was measured on immunoblots by use of anti-phospho-Thr172 AMPK (Cell Signaling), anti-phospho-Ser473 and anti-phospho-Thr308 PKB/Akt (Cell Signaling), anti-phospho-Thr389 p70S6K (Santa Cruz Biotechnology), anti-phospho-Tyr1150/1151 IR (Cell Signaling), anti-phospho-Ser636/639 IRS-1 (Cell Signaling), and anti-phospho-Tyr612 IRS-1 (Invitrogen) antibodies, respectively. Phosphorylation state of AS160 was measured using the anti-phospho-Akt substrate (anti-PAS; Cell Signaling) antibody. Total amount of...
AMPK, PKB/Akt, p70S6K, AS160, IR, and IRS-1 was verified by immunoblotting using anti-AMPK (Cell Signaling), anti-PKB/Akt (Upstate), anti-p70S6K (Cell Signaling), anti-AS160 (Upstate), anti-IR (Upstate), and anti-IRS-1 (Upstate) antibodies, respectively. For each gel, control of loading was realized by immunoblotting using an anti-eukaryotic elongation factor 2 (eEF2) antibody (Cell Signaling) after the membranes were stripped. Band intensities were quantified by scanning and processing with the program ImageJ (1.33 for Mac OS X). The quantification of immunoblots presented in RESULTS was obtained after normalization using the internal eEF2 loading control. The quantification of immunoblots given by the anti-phospho antibody has been double normalized by the quantification of a loading control (anti-eEF2 on the same gel after stripping) and by the quantification given by the respective anti-total antibody (also normalized with the loading control) made on another gel.

Protein measurement and statistics. Protein content was estimated by the method of Bradford with BSA as a standard. The results are expressed as means ± SE. The statistical significance was calculated using two-tailed unpaired Student’s test and one-way ANOVA using Bonferroni post hoc test for simple and multiple comparisons, respectively.

RESULTS

AMPK activation increases insulin signaling by inhibiting p70S6K. Primary cultured cardiomyocytes were incubated with insulin (at physiological 3 × 10⁻⁹ M concentration), phenformin (0.5 mM), or a combination of both (Fig. 1). As previously shown (4), phenformin induced the phosphorylation of AMPK (Fig. 1B), whereas insulin promoted that of PKB/Akt (Fig. 1, C and D). When added together, phenformin further increased the insulin-induced PKB/Akt phosphorylation on both Thr-308 and Ser-473 residues (Fig. 1, C and D). This insulin-sensitizing effect of AMPK activators was similar to that previously described (4).

The phosphorylation state of p70S6K on Thr-389, which is known to correlate with the activity of the mTOR/p70S6K pathway, was measured in cardiomyocytes treated as described above (Fig. 2A). In agreement with previous data obtained in perfused hearts and cardiomyocytes in suspension (2, 16), no p70S6K phosphorylation could be detected in untreated cells and AMPK activation by phenformin had no effect under these basal conditions (Fig. 2A). However, when p70S6K was phosphorylated as a result of insulin treatment, AMPK activation by phenformin significantly decreased (±30% of inhibition) this insulin-induced Thr-389 phosphorylation (Fig. 2A). We, then, evaluated the phosphorylation state of IRS-1 on Ser-636/639, the target of the mTOR/p70S6K pathway in the negative feedback loop. Insulin treatment induced IRS-1 phosphorylation on Ser-636/639 (Fig. 2B), which was substantially inhibited (±50%) by a cotreatment with the AMPK activator phenformin (Fig. 2B).

In our previous study (4), we showed that oligomycin, an AMPK activator unrelated to any antidiabetic action, increased the insulin-induced PKB/Akt phosphorylation and glucose uptake stimulation, mimicking phenformin effect. Similarly to phenformin, oligomycin repressed the insulin-mediated phosphorylation of p70S6K and IRS-1 (on Ser-636/639) and over-stimulated PKB/Akt (Fig. 3).

Effect of rapamycin on the insulin-induced negative feedback loop. Next, we evaluated the ability of rapamycin to mimic AMPK activators on insulin action (Figs. 1 and 2). Rapamycin incubation did not modify the AMPK phosphorylation (Fig. 1B), but like AMPK activators, this mTOR/p70S6K inhibitor induced the insulin-mediated overphosphorylation of PKB/Akt (Fig. 1, C and D), which correlated with the decrease of the insulin-induced phosphorylation of p70S6K on Thr-389 (Fig. 2A) and of IRS-1 on Ser-636/639 (Fig. 2B). Actually, the stimulation of PKB/Akt phosphorylation by rapamycin was even greater than that by phenformin (Fig. 1, C and D). This could be explained by the fact that rapamycin is a more potent inhibitor of the mTOR/p70S6K/IRS-1 negative feedback loop (Fig. 2, A and B).

Effect of phenformin and rapamycin on the initial steps of insulin signaling. Even if the decrease in IRS-1 phosphorylation on Ser-636/639 is sufficient to explain the AMPK-mediated overstimulation of PKB, it was essential to verify that the more proximal insulin signaling was unchanged. Therefore, we
measured the phosphorylation state of the insulin receptor and IRS-1 on tyrosine residues (Fig. 4, B and C). As expected, insulin induced the auto-transphosphorylation of the insulin receptor on Tyr-1150/Tyr-1151. This resulted in the increase of IRS-1 phosphorylation on Tyr-612. Neither phenformin nor rapamycin modified these insulin-mediated phosphorylations on tyrosine.

Effect of insulin, AMPK activators, and rapamycin on AS160 phosphorylation and glucose uptake. The phosphorylation state of AS160 is believed to mediate, at least in part, the effect of insulin and AMPK on glucose uptake (5). Indeed, both PKB/Akt and AMPK are able to phosphorylate and inhibit AS160. Knowing that AS160 inhibits GLUT4 translocation, its inhibition by PKB/Akt and AMPK promotes glucose uptake (Fig. 5A). AS160 phosphorylation state was measured in the different conditions used in the present study. In agreement with previous studies (25, 31), insulin induced AS160 phosphorylation in cardiomyocytes (Fig. 5B). Oligomycin and phenformin also promoted AS160 phosphorylation (Figs. 3 and 5B). In line with the effect of AMPK activators on the insulin-depandent PKB/Akt phosphorylation, phenformin, when added together with insulin, further increased AS160 phosphorylation (±3-fold increase). Rapamycin, alone, did not modify AS160 phosphorylation. In the presence of insulin, it increased the phosphorylation of AS160, although to a lesser extend than phenformin (±2-fold increase). Concerning glucose uptake, we confirmed that the PKB/Akt overstimulation found in the presence of both insulin and AMPK activator correlated with a synergistic effect on glucose uptake (7-, 5-, and 17-fold increase for insulin, phenformin and insulin + phenformin treatments, respectively; Fig. 5C). In contrast to this synergistic action of phenformin, rapamycin had no effect on insulin-stimulated glucose uptake despite its effect on PKB/Akt/AS160 phosphorylation (Fig. 5C).

Effect of phenformin and rapamycin on a dose-response curve of insulin. The disconnection between the rapamycin action on insulin signaling and its lack of effect on glucose uptake was confirmed at several insulin concentrations (Fig. 6). First of all, phenformin was able to induce AMPK phosphorylation at all concentrations of insulin (Fig. 6A). As the other dose-response curves showed, all effects of insulin on its downstream elements were maximal at about $10^{-9}$ M insulin (Fig. 6, B-F). In agreement of the results presented at Figs. 1 to 3 and 5, the effect of insulin on 1) PKB/Akt phosphorylation was amplified in the presence of phenformin or rapamycin (Fig. 6B); 2) p70S6K (Fig. 6C) and IRS-1 (Fig. 6D) phosphorylation was partially blocked by phenformin and abrogated in the presence of rapamycin; and 3) AS160 phosphorylation was increased by phenformin and, to a lesser extend, by rapamycin (Fig. 6E). Finally, whereas phenformin stimulated glucose uptake (maximum 5.5-fold) and amplified insulin action on this metabolic pathway (maximum 8-fold and 16-fold for insulin and insulin + phenformin, respectively), rapamycin had no effect at any concentration of insulin (Fig. 6F).
scribed previously (4). As in insulin-sensitive cells, rapamycin reproduced the phenformin effect on PKB/Akt phosphorylation. The increase in insulin sensitivity induced by both phenformin and rapamycin in insulin-resistant cardiomyocytes was highlighted at the insulin concentration of 3 × 10^{-9}M (Fig. 7B, inset). At this concentration, insulin was unable to induce any significant PKB/Akt phosphorylation whereas phenformin and rapamycin restored insulin action on this target. In parallel to PKB/Akt, the insulin-dependent p70S6K phosphorylation curve was also shifted to the right (Fig. 7C). This phosphorylation was inhibited by phenformin and blocked by rapamycin. The phosphorylation of IRS-1 on Ser-636/639 was characterized by the same pattern (Fig. 7D). As for the previous elements of the insulin signaling, the phosphorylation of AS160 by this hormone was severely impaired in insulin-resistant cardiomyocytes (Fig. 7E). Phenformin, alone, increased the phosphorylation state of AS160 and substantially enhanced insulin action. On the other hand, rapamycin, alone, did not affect AS160 phosphorylation and intensified the effect of insulin but only at the highest concentration of this hormone. Concerning the metabolic effect, insulin failed to normally stimulate glucose uptake
under insulin-resistant conditions (Fig. 7F). A significant stimulation was only obtained at $10^{-8}$ M and reached a level fivefold higher than that found in untreated cardiomyocytes. As in insulin-sensitive cells, phenformin, alone, induced a sixfold increase in glucose uptake and remarkably amplified insulin action restoring a synergistic effect similar to that seen in normal cells (20-fold increase). By contrast, rapamycin had clearly no action on the insulin-mediated stimulation of glucose uptake. To summarize, the effects of rapamycin were similar in insulin-sensitive than in insulin-resistant cardiomyocytes, i.e., a clear amplification of insulin signaling, which contrasts with its lack of effect on glucose uptake.

**DISCUSSION**

The aim of this work was to verify, in the heart, the generally accepted hypothesis that inhibition of the mTOR/p70S6K pathway by AMPK is responsible for the insulin-sensitizing action of AMPK on insulin signaling and glucose uptake.

AMPK activation enhances insulin action on cardiac PKB/Akt by inhibiting the negative feedback loop mediated by mTOR/p70S6K. We report here that AMPK activation by the biguanide phenformin reduced the insulin-dependent phosphorylation of p70S6K (on Thr-389) and of IRS-1 (on Ser-636/639), which clearly correlated with an increase in PKB/Akt phosphorylation in both insulin-sensitive and insulin-resistant cardiomyocytes. The activation of AMPK by another activator unrelated to any antidiabetic function, namely oligomycin, gives a similar response. Moreover, this effect could be mimicked by rapamycin, a specific inhibitor of the mTOR/p70S6K pathway. Taken together, these results demonstrate that inhibition of the insulin negative feedback on insulin signaling is responsible of the insulin-sensitizing effect of AMPK activators on PKB/Akt in cardiomyocytes (Fig. 8). The demonstration that activation of AMPK by adiponectin in C2C12 myotubes has an insulin-sensitizing effect on PKB/Akt phosphorylation by inhibiting the p70S6K/IRS-1 axis (39) is in line with our results.

Knowing that there is an opposite action of PKB/Akt (activation) and AMPK (inactivation) on p70S6K, one may wonder...
Fig. 7. Insulin dose-response curve in the presence or not of phenformin and rapamycin on AMPK (A), PKB/Akt (B), p70S6K (C), IRS-1 (D), AS160 (E) phosphorylation state and glucose uptake (F) in insulin-resistant cardiomyocytes. Cardiomyocytes were incubated as described in Fig. 1. Values for phosphorylation state are relative to the positive control (phenformin alone for AMPK and insulin alone for PKB/Akt, p70S6K, IRS-1, and AS160). B, inset: PKB phosphorylation state obtained at a concentration of $3 \times 10^{-9}$ M of insulin. Values are the means $\pm$ SE of at least 3 experiments. $p < 0.05$ vs. insulin alone.

Fig. 8. Proposed scheme of the regulation of glucose uptake by insulin and AMPK in the cardiomyocytes. Downstream insulin and its receptor (IR), the phosphorylation of IRS-1 on tyrosine residues leads to the phosphorylation and activation of PKB/Akt, which then phosphorylates and inhibits AS160 allowing the translocation of the glucose transporter GLUT4 storage vesicles to the plasma membrane (1). In addition, PKB/Akt induces the phosphorylation and activation of the mammalian target of rapamycin (mTOR)/p70S6K pathway, which, then, phosphorylates IRS-1 on serine residues leading to the inhibition of the insulin signaling (2). AMPK phosphorylates and inhibits AS160 stimulating glucose uptake independently of insulin (3). AMPK also inhibits the mTOR/p70S6K-dependent negative feedback loop leading to the overphosphorylation of the PKB/Akt/AS160 axis (4). Part of the insulin action on glucose uptake is independent of PKB/Akt/AS160 (5). The insulin-sensitizing effect of AMPK on glucose uptake could be due to the action of AMPK on these events (6).
why the resulting action of insulin and phenformin, when incubated together, is the decrease in p70S6K phosphorylation. This preferential action of AMPK could be explained by two phenomena. First, AMPK acts downstream PKB/Akt in the regulation of the mTOR/p70S6K pathway (13). Second, we intentionally preincubated cardiomyocytes with AMPK activators to be certain that AMPK was already activated before treating cells with insulin.

Inhibition of the mTOR/p70S6K-mediated negative feedback loop is not involved in the insulin-sensitizing effect of AMPK activators on cardiac glucose uptake. In the present study as in our previous work (4), we showed that AMPK activation overstimulated the insulin-induced glucose uptake, this effect being more visible in insulin-resistant cardiomyocytes. By contrast, rapamycin was unable to improve the insulin-dependent glucose transport despite clear PKB/Akt overphosphorylation. In agreement with our results, rapamycin treatment was reported to fail to improve glucose or insulin tolerance in ob/ob mice although mTOR/p70S6K activity was decreased (23). Therefore, we concluded that 1) inhibition of the mTOR/p70S6K-mediated negative feedback on insulin signaling has no bearing on the metabolic effect (glucose uptake) of insulin in the heart; and 2) PKB/Akt activation is not a rate-limiting step in the regulation of cardiac glucose uptake, because its overphosphorylation has no effect on the metabolic rate. This evidence contradicts the prevailing concept, namely that inactivation of mTOR/p70S6K improves insulin sensitivity and is one of the main molecular mechanisms involved in the increase of the insulin-mediated glucose uptake (21, 32, 34). Moreover, our results also underline the importance of measuring metabolic effects and demonstrate the shortcomings of taking PKB/Akt as the sole index of insulin action (39). Several other molecular mechanisms that can be targeted by both insulin and AMPK and are involved in the regulation of glucose uptake, could explain the effects presented in the present study (Fig. 8). It is known that distal GLUT4 trafficking event downstream of insulin is controlled by actin polymerization and by v- and t-soluble N-ethylmaleimide-sensitive factor association protein receptors (SNAREs; Refs. 5, 22). Knowing that these actors are also regulated by AMPK (20, 24, 30), it is tempting to postulate a possible action of one or several of these partners in the effect of AMPK activators in the insulin-mediated stimulation of glucose uptake. For instance, the fact that the insulin-induced actin cytoskeleton rearrangement is impaired in insulin-resistant conditions (18) reenforces such hypothesis. This needs further investigations.

It should be mentioned that, at variance with our data, rapamycin was found to increase insulin-dependent glucose uptake in both insulin-sensitive and insulin-resistant 3T3-L1 adipocytes (3, 28, 35). This discrepancy suggests that the effect of mTOR/p70S6K inhibition on glucose uptake could be cell or tissue specific. This is reenforced by the fact that the deletion of p70S6K in mice protects against diet-induced obesity and enhances insulin sensitivity (37).

AS160, a point of convergence between insulin and AMPK signaling. We confirmed that AS160 can be regulated by insulin and AMPK activators, the association of both agents inducing an overphosphorylation of AS160 as previously shown (1, 31). The implication of mTOR/p70S6K inhibition in this insulin-sensitizing action of AMPK activators on AS160 phosphorylation was established by the use of rapamycin, which also causes an overphosphorylation of AS160. The effect of rapamycin is, nevertheless, less important than that of AMPK activators. Indeed, the action of AMPK activators on AS160 phosphorylation results from the addition of their insulin-independent (direct phosphorylation by AMPK) and insulin-sensitizing (overstimulation of PKB/Akt) effects, whereas rapamycin action is restricted to its insulin-sensitizing effect.

Is AS160 phosphorylation controlling insulin-mediated glucose uptake? The most significant information of the present study concerning AS160 is that, like for PKB/Akt, increasing its phosphorylation state under rapamycin treatment does not increase glucose uptake. This emphasizes the disconnection between the PKB/Akt pathway and glucose uptake downstream of insulin. In other words, PKB/Akt/AS160 does not seem to be the rate-limiting step in the control of glucose uptake by insulin in the heart. A divergence between PKB/Akt/AS160 signaling and glucose uptake was similarly shown in a model of diet-induced obesity where the insulin-induced glucose uptake was reduced whereas PKB/Akt/AS160 signaling was preserved (40).

Conclusions. From our study, we conclude that 1) the inhibition of the mTOR/p70S6K-mediated negative feedback loop is not sufficient to increase the insulin sensitivity in terms of metabolic effect in the heart; 2) the PKB/Akt/AS160 pathway is not a rate-limiting step in the regulation of cardiac glucose uptake downstream of insulin; and 3) other(s) hitherto unknown mechanism(s) could be involved in the insulin-sensitizing effect of AMPK activators on the insulin-stimulated cardiac glucose uptake.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


