FEBS Letters 505 (2001) 348–352 FEBS 25194

Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides

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Received 9 July 2001; revised 3 August 2001; accepted 3 August 2001

First published online 14 August 2001

Edited by Jacques Hanoune

Abstract AMP-activated protein kinase (AMPK) is known to be activated by phosphorylation on Thr172 in response to an increased AMP/ATP ratio. We report here that such an activation indeed occurred in anaerobic rat hearts and that it was antagonized (40–50%) when the hearts were pre-treated with 100 nM insulin. The effect of insulin (1) was blocked by wortmannin, an inhibitor of phosphatidylinositol-3-kinase; (2) only occurred when insulin was added before anoxia, suggesting a hierarchical control; (3) resulted in a decreased phosphorylation state of Thr172 in AMPK and (4) was unrelated to changes in the AMP/ATP ratio. This is the first demonstration that AMPK activity could be changed without a detectable change in the AMP/ATP ratio of the cardiac cell.

Key words: AMP-activated protein kinase; Heart; Ischemia; Anoxia; Insulin

1. Introduction

AMP-activated protein kinase (AMPK) is a well-conserved eukaryotic protein kinase that senses the energy state of the cell. It is activated under anaerobic conditions and acts as a metabolic master switch [1]. AMPK is a heterotrimeric protein composed of a catalytic subunit (α) and two regulatory subunits (β and γ). Isoforms of all three subunits are known. Their subcellular location differs but their respective roles remain unclear [2,3]. In the heart, the α_2 isoform of the catalytic subunit is more abundant than α_1 [4].

The control of AMPK activity is complex. It involves allosteric stimulation by AMP as well as phosphorylation [1]. AMP allosterically stimulates both AMPK and AMPK-kinase (AMPKK), the upstream kinase. In addition, AMP promotes AMPK phosphorylation by AMPKK and prevents its dephosphorylation by protein phosphatase 2C. Activation of AMPK results from phosphorylation of Thr172 in the activation loop of the catalytic α-subunit, although other phosphorylation sites have been reported [5]. ATP is a competitive inhibitor with regards to AMP with, however, a more than 10-fold lower affinity [1]. Therefore, in normoxic hearts, ATP (5–10 mM) antagonizes the effect of the low AMP concentration (0.1–0.2 mM) and keeps AMPK mainly in the inactive dephosphorylated form. By contrast, when the energy state of the cell collapses as in ischemic or anoxic hearts, the AMP/ATP ratio increases and activates AMPK 5–10-fold [6,7]. AMPK is also activated allosterically by a decreased phosphocreatine/creatine (PCr/Cr) ratio in muscle [8]. This reinforces the activity-dependence of AMPK on the energy state of the cell.

In hearts submitted to anaerobic conditions, AMPK activation participates in the stimulation of glycolysis by enhancing the recruitment of GLUT4 [9] to the plasma membrane and by activating 6-phosphofructo-2-kinase [7]. This enzyme synthesizes fructose 2,6-bisphosphate, a potent positive allosteric effector of 6-phosphofructo-1-kinase, a key glycolytic enzyme. AMPK also inactivates acetyl-CoA carboxylase (ACC) [10]. ACC inactivation might persist during reperfusion following an ischemic episode, and so contributes to a stimulation of fatty acid oxidation during this period [11]. Indeed, ACC activity controls the concentration of malonyl-CoA, which is a potent inhibitor of the entry of long-chain fatty acids into mitochondria. Phosphorylation and activation of malonyl-CoA decarboxylase, a recently discovered substrate of AMPK, exerts the same overall effect on fatty acid metabolism [12]. In normoxic hearts, AMPK has been reported to be inactivated by insulin [13,14]. This effect was proposed to mediate ACC activation and inhibition of fatty acid oxidation by insulin. The mechanism involved in the control of AMPK activity by insulin is unknown.

In this work, we studied the effect of insulin to antagonize AMPK activation in rat hearts submitted to ischemia or anoxia. Insulin was found to inactivate AMPK by decreasing the phosphorylation state of Thr172 by a mechanism unrelated to changes in the AMP/ATP and PCr/Cr ratios. This is the first report that AMPK activity was changed without detectable change in the AMP/ATP ratio of the cardiac cell.

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Abbreviations: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; PCr, phosphocreatine; Cr, creatine; ACC, acetyl-CoA carboxylase; PI3K, phosphatidylinositol-3-kinase

2. Materials and methods

2.1. Perfusion protocol

Hearts from male Wistar rats (200–220 g body weight, anesthetized with 50 mg/kg pentobarbital injected intraperitoneally) were perfused [15] retrogradely at a constant pressure of 60 mm Hg with a Krebs

0014-5793/01/$20.00
PII: S0014-5793(01)02788-0

Henseleit bicarbonate buffer containing 1.5 mM $CaCl_2$ and 5 mM glucose and in equilibrium with a 95% O_2/5% CO_2 gas phase, if not otherwise stated. No-flow ischemia was obtained by interrupting the flow. Anoxia was obtained by replacing O_2 by N_2 in the gas phase. Hearts were freeze-clamped at the times indicated in the figures.

2.2. AMPK activity and phosphorylation state

The frozen hearts were homogenized (Ultra-Turrax) at 0–4°C in 5 (v/w) vol of lysis buffer (50 mM HEPES at pH 7.5, 50 mM KCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM β-glycerolphosphate, 100 nM microcystin-LR, and protease inhibitors (4 μg/ml leupeptin, 1 mM benzamidine hydrochloride, 0.7 μg/ml pepstatin, 0.2 μM phenylmethylsulfonyl fluoride and 0.4 μg/ml trypsin inhibitor)) and the supernatants (10 000 × *g*, 30 min) were stored at −80°C [7]. AMPK activity was assayed in the presence of 0.2 mM AMP in a 10% polyethylene glycol 6000 fraction [7,16]. 1 unit of protein kinase activity corresponds to the formation of 1 nmol of product per minute under the assay conditions. AMPK phosphorylation state was determined by immunoblotting (anti-phosphoThr172 AMPK antibody) after immunoprecipitation with anti-α_1 and/or anti-α_2 AMPK antibodies [17,18]. Proteins were measured by Coomassie blue staining with bovine serum albumin as a standard.

2.3. Metabolites

AMP, ADP and ATP were measured in neutralized perchloric acid extracts of the frozen hearts after their separation by high-performance liquid chromatography (HPLC) [19]. PCr, Cr and lactate were measured enzymatically in the same extracts, as in [20]. It should be noted that adenine nucleotides and (P)Cr were measured in deproteinized extracts of whole tissue, and so any compartmentation was not taken into account.

3. Results

AMPK activity was measured in hearts submitted to periods of no-flow ischemia, ranging from 0 to 20 min (Fig. 1A). The basal AMPK activity was low, as expected for normoxic conditions. AMPK activity increased progressively during the

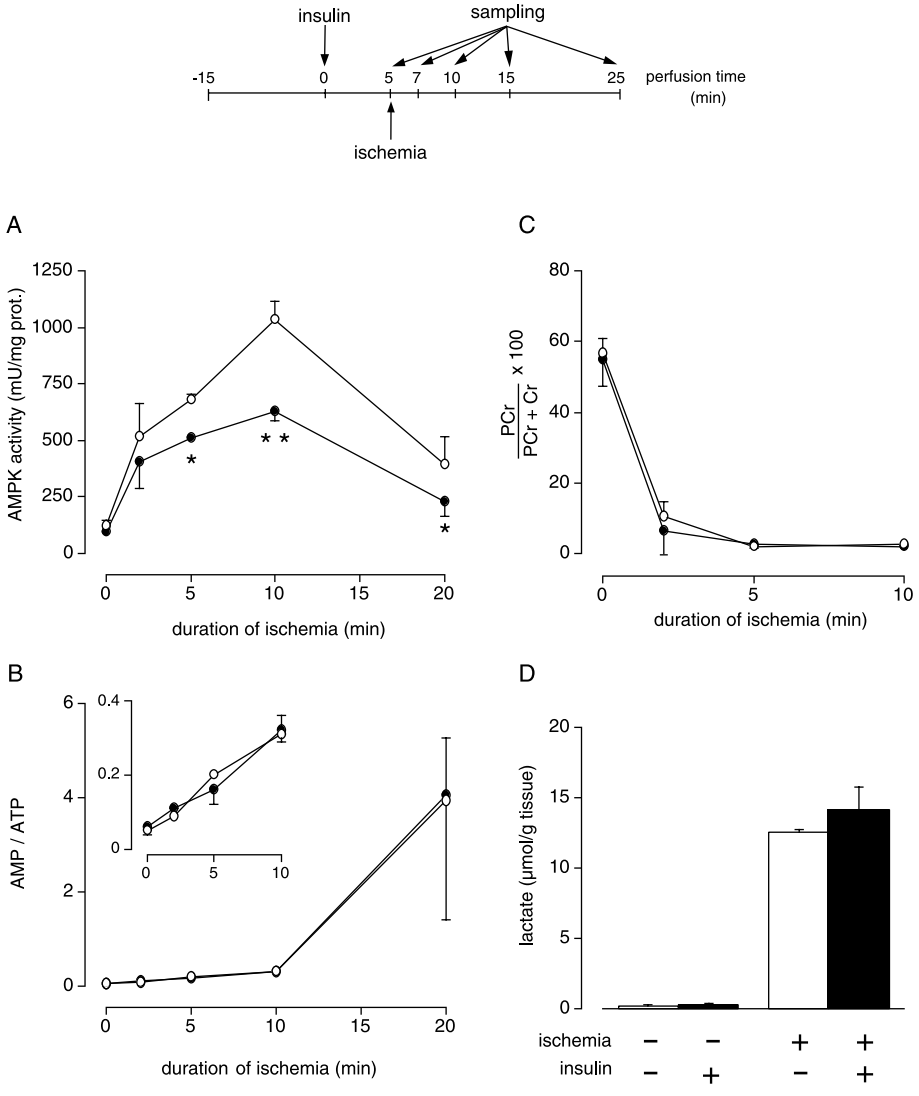

Fig. 1. Effect of insulin pre-treatment on AMPK activity (A), AMP/ATP (B) and PCr/Cr (C) ratios, and lactate accumulation (D) in rat hearts submitted to no-flow ischemia. A scheme of the experimental protocol and the times of sampling are given at the top of the figure. After 15 min equilibration, the hearts were perfused for 5 min with insulin (100 nM) before ischemia was started. B: The inset represents the AMP/ATP ratio during the first 10 min ischemia. The absolute values (μmol/g wet tissue) ranged from 2.67 ± 0.16 to 0.43 ± 0.16 for ATP, from 0.90 ± 0.12 to 0.47 ± 0.07 for ADP, from 0.14 ± 0.02 to 1.26 ± 0.16 for AMP, from 4.94 ± 0.23 to 0.22 ± 0.08 for PCr and from 8.5 ± 0.5 to 3.76 ± 0.42 for Cr (control vs. 20 min ischemia, without insulin). D: Lactate content (μmol/g wet tissue) was measured in hearts submitted to 10 min no-flow ischemia. Open bars: control hearts; closed bars: insulin pre-treated hearts. The values are the means ± S.E.M. of four hearts. $^*P < 0.05$ indicates values that are statistically different from the corresponding values at the same perfusion times.

ischemic episode to reach a 10-fold activation after 10 min. The activation was transient and AMPK activity returned towards basal values between 10 and 20 min. Pre-treatment for 5 min with 100 nM insulin antagonized the activation of AMPK during ischemia at 5 min and onwards. The inhibition was maximal (40%) at 10 min ischemia.

Changes in AMPK activity are known to result from alterations in AMP/ATP as well as PCr/Cr ratios. AMPK activation strictly correlated with changes in the AMP/ATP (Fig. 1B) and PCr/Cr (Fig. 1C) ratios during the first 10 min ischemia. One can calculate that, between 2 and 10 min ischemia, AMPK activity doubled (Fig. 1A) for an increase in the AMP/ATP ratio of about 0.3 (inset Fig. 1B). However, the relationship between AMPK activity and AMP/ATP ratio did not hold true for the last 10 min ischemia, when the AMP/ATP ratio drastically increased (Fig. 1B), whereas AMPK activity decreased (Fig. 1A). Importantly, insulin did not affect the AMP/ATP ratio at any time (Fig. 1B), although it did inhibit AMPK activation (Fig. 1A). From the relationship between changes in AMPK activity and changes in the AMP/ATP ratio in ischemic hearts, one can calculate that the change in AMPK activity brought about by insulin (400 mU/mg protein) should have corresponded to a change of about 0.2 in the AMP/ATP ratio. Such an easily detectable change in the AMP/ATP ratio was obviously not found. Moreover, insulin was without effect on the large decrease in the PCr/Cr ratio observed after 2 min ischemia (Fig. 1C), and it did not modify the glycolytic rate as indicated by lactate accumulation in the ischemic hearts (Fig. 1D). This demonstrated that insulin could change AMPK activity without affecting the energy state of the ischemic hearts.

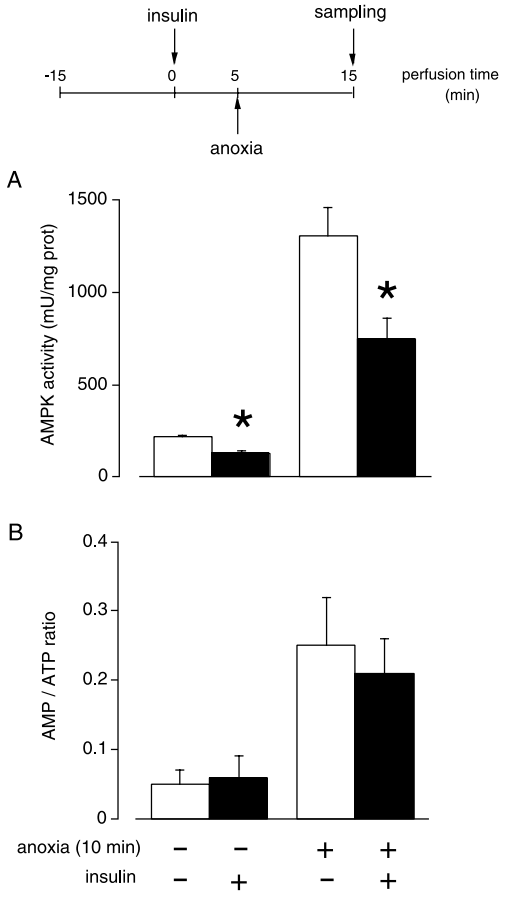

Fig. 2. Effect of insulin pre-treatment on AMPK activity (A) and AMP/ATP ratio (B) in rat hearts submitted to anoxia. A scheme of the experimental protocol and the times of sampling are given at the top of the figure. After 15 min equilibration, the hearts were perfused for 5 min with insulin (100 nM) before anoxia was started. The hearts were freeze-clamped after 10 min anoxia. Open bars: control hearts; closed bars: insulin pre-treated hearts. The values are the means ± S.E.M. of at least four hearts. $*P < 0.05$ indicates values that are statistically different from the corresponding non-insulinic hearts.

The energy state-independent antagonism between insulin and AMPK was confirmed in hearts submitted to a 10 min episode of anoxia (Fig. 2). Under these conditions, the inhibition of AMPK activation by insulin (50%) was slightly larger than in ischemia (Fig. 2A). In anoxia, like in ischemia, insulin did not change the AMP/ATP ratio (Fig. 2B). In agreement with previous studies [14], we also observed a small but significant effect of insulin on AMPK activity in normoxic hearts.

Insulin did not modify AMPK activity when it was added 5 min after the beginning of anoxia, i.e. when AMPK was already activated (Fig. 3). The lack of insulin effect was confirmed for durations of anoxia (Fig. 3A) or insulin exposure (Fig. 3B) that were the same as in Fig. 2, when insulin was added before anoxia.

To know whether the insulin-induced mechanism could be mediated by changes in the phosphorylation state of AMPK, we resorted to immunoblots using an antibody raised against a peptide corresponding to the amino acid sequence surrounding the phosphorylated Thr172 in AMPK. A 10 min episode of ischemia or anoxia increased Thr172 phosphorylation of both isoforms of the catalytic subunit of AMPK (Fig. 4A). Insulin antagonized this phenomenon, its effect being more important in anoxia than in ischemia, consistent with the slight difference in AMPK activity (compare Figs. 1A and 2A). The effect of insulin was confirmed separately for each isoform (Fig. 4B).

The anti-AMPK effect of insulin during anoxia was completely inhibited in the presence of 0.3 μM wortmannin, a known inhibitor of phosphatidylinositol-3-kinase (PI3K; Fig. 5). This anti-AMPK effect was unaffected by 0.25 μM rapamycin, an inhibitor of p70 ribosomal S6 kinase activation (Fig. 5).

4. Discussion

This work shows that insulin inhibited the activation of AMPK resulting from ischemia or anoxia. The inhibitory effect of insulin corresponded to a decreased phosphorylation state of Thr172 in the activation loop of the catalytic subunit of AMPK. This is the first experimental evidence that AMPK activity can be modified without change in the total adenine nucleotides concentration. In agreement with this finding, perfusion of rat hearts with non-esterified fatty acids has recently been found to activate AMPK without affecting the AMP/ATP ratio (E.D. Saggerson and H.S. Clark, personal communication). Moreover, in our ischemic models, insulin did not affect the PCr/Cr ratio, which is known to be sensitive to small and rapid changes in the energy state of the cytosol during an anaerobic episode. Insulin was also without effect on lactate accumulation and hence on glycolysis, which is already stimulated under these anaerobic conditions. This is in

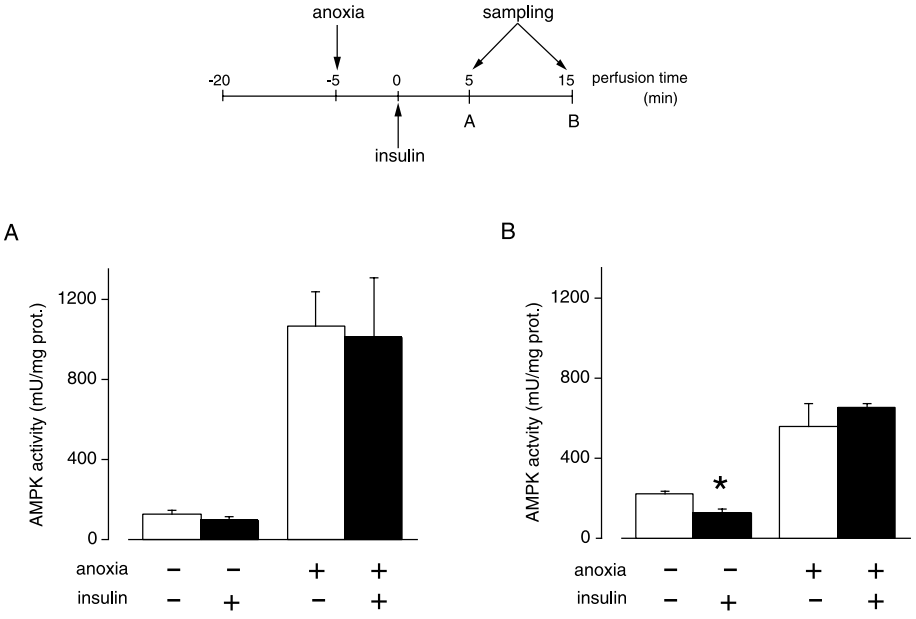

Fig. 3. AMPK activity in anoxic hearts treated with insulin after the beginning of anoxia. A scheme of the experimental protocol and the times of sampling are given at the top of the figure. After 15 min equilibration, the hearts were submitted to anoxia. Insulin (100 nM) was added 5 min after the beginning of anoxia. The hearts were freeze-clamped after 10 min (A) or 20 min (B) anoxia. Open bars: control hearts; closed bars: insulin pre-treated hearts. The values are the means ± S.E.M. of at least four hearts. $*P < 0.05$ indicates values that are statistically different from the corresponding non-insulinic hearts.

line with the previously reported lack of an additive effect of insulin and severe ischemia on glucose metabolism in the heart [21,22]. Therefore, no change in the AMP/ATP ratio is expected in the cytosolic compartment where glycolysis operates.

The anti-AMPK effect of insulin was only observed when insulin was given before the anaerobic episode. Moreover, we previously demonstrated that ischemia inhibits insulin signaling [23]. Therefore, both observations suggest that the effect of insulin on the AMPK cascade was initiated in normoxic hearts before the anaerobic episode. This priming of the hearts by insulin pre-treatment prevented the phosphorylation and activation of AMPK brought about by ischemia or anoxia.

The decreased phosphorylation state of Thr172 could result either from an inactivation of AMPKK, from the phosphorylation of AMPK on a site that prevents Thr172 phosphorylation, or, alternatively, from the activation of a phosphatase. However, the latter is considered to be unlikely, because it is not consistent with the fact that insulin action should precede the anaerobic AMPK activation. Whatever the mechanism involved, insulin action could result in a decreased sensitivity of the AMPK/AMPKK system towards changes in adenine nucleotide concentration.

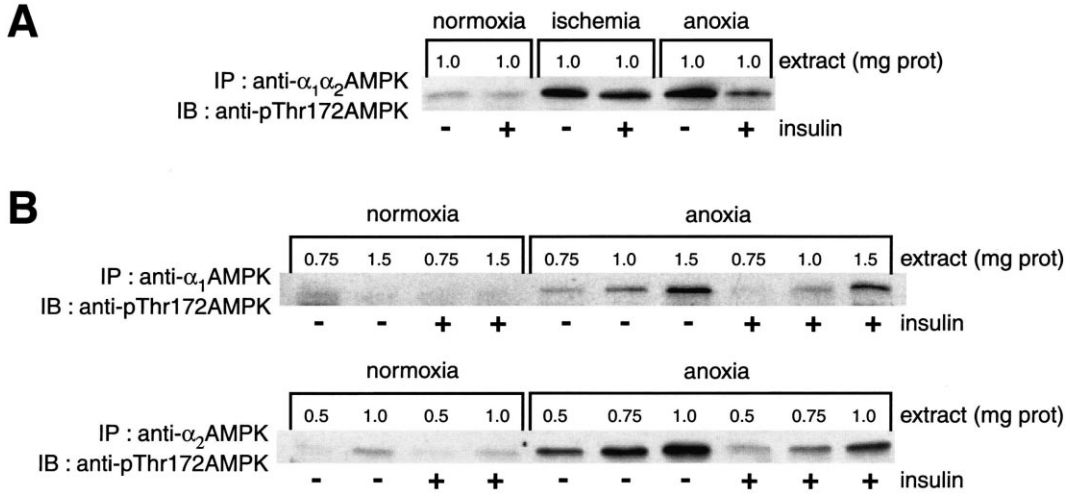

Fig. 4. Effect of insulin pre-treatment on the phosphorylation state of Thr172 after 10 min ischemia or anoxia. A: The catalytic subunits (α_1 and α_2) were co-immunoprecipitated (IP: anti-$\alpha_1\alpha_2$AMPK). B: The catalytic subunits were immunoprecipitated separately (IP: anti-α_1AMPK or anti-α_2AMPK). The amounts of heart extract used for the immunoprecipitation are indicated. The phosphorylation state was compared by immunoblots with anti-phosphoThr172 AMPK antibody (IB: anti-pThr172AMPK). The amount of AMPK present in the extract was the same as measured by immunoblots with anti-α_2AMPK (not shown). The blots shown are representative of three different experiments.

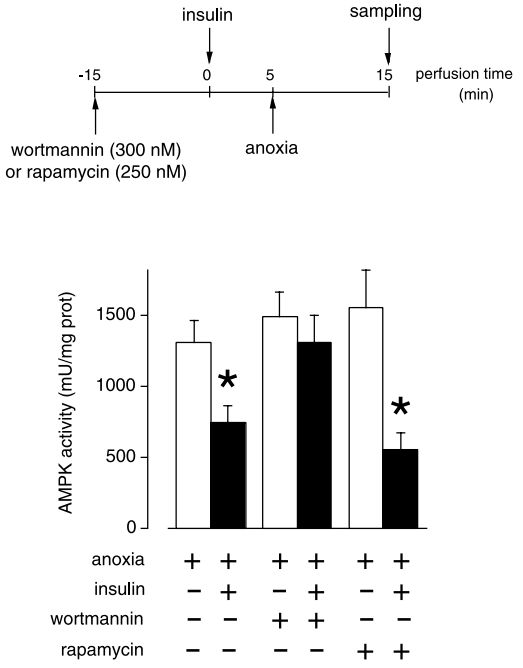

Fig. 5. Effect of wortmannin and rapamycin on the insulin-induced inhibition of AMPK activation after 10 min of anoxia. A scheme of the experimental protocol and the time of sampling are given at the top of the figure. The values are the means ± S.E.M. of at least six hearts. $*P < 0.05$ indicates values that are statistically different from the corresponding non-insulinic hearts.

The anti-AMPK effect of insulin was wortmannin-sensitive, like most short-term effects of insulin, suggesting that it is mediated by PI3K. According to our current understanding of insulin action, a cross-talk between the AMPK cascade and a step of the insulin signaling pathway located downstream of PI3K has not been reported and is certainly worth investigating.

AMPK activity decreased between 10 and 20 min ischemia, although the AMP/ATP ratio still increased about 10-fold. This may be related to a progressive decrease in the concentration of ATP, which is a substrate for AMPKK. Moreover, it is likely that the large increase in AMP competes with the small amount of ATP remaining, and so inhibits phosphorylation of AMPK by AMPKK. The transient activation of AMPK could also reflect the occurrence of a negative feedback mechanism.

The effect of insulin was readily observed under anaerobic conditions, when AMPK activity is already elevated. It was less evident under normoxic conditions, although it has been originally reported for such conditions [14]. In normoxic conditions, the metabolic consequences of the interaction between insulin and AMPK would be to increase malonyl-CoA concentrations. This would eventually limit fatty acid oxidation and is in line with the observation that insulin favors glucose utilization and restricts fatty acid oxidation. The absence of oxygen should re-inforce this effect of insulin to inhibit fatty acid oxidation. Under the same conditions, although insulin inhibits AMPK, the stimulation of glycolysis should remain elevated, because both ischemia and insulin stimulate glycolysis by activating the same key steps [7,9,21], namely the recruitment of GLUT4 and the activation of 6-phosphofructo-2-kinase. Therefore, we speculate that the physiological relevance of insulin counteracting AMPK activation in ischemic hearts concerns other targets of this kinase, as yet unknown. It is also worth considering whether this anti-AMPK effect is related to the protective role of insulin against ischemia-reperfusion injuries in the heart.

Acknowledgements: The assistance of L. Maisin, V. O'Connor and P. Durante is greatfully acknowledged. We thank M.H. Rider for his interest and critical reading of the manuscript and A. Delacauw for her help with HPLC. We also thank the Belgian Fund for Scientific Research for the financial support of C.B., A.-S.M. and L.B. This work was supported by the 'Actions de Recherche Concertées' 98/03-216 (French Community of Belgium) and by the Belgian Fund for Medical Scientific Research. U.K. was supported by the Belgian Federal Programme Interuniversity Poles of Attraction (P4/23) and D.G.H. by a programme grant from the Welcome Trust.

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