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The stimulation of heart glycolysis by increased workload does not require AMP-activated protein kinase but a wortmannin-sensitive mechanism

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Abstract Increasing heart workload stimulates glycolysis by enhancing glucose transport and fructose-2,6-bisphosphate (Fru-2,6-P_2), the latter resulting from 6-phosphofructo-2-kinase (PFK-2) activation. Here, we investigated whether adenosine monophosphate (AMP)-activated protein kinase (AMPK) mediates PFK-2 activation in hearts submitted to increased workload. When heart work was increased, PFK-2 activity, Fru-2,6-P_2 content and glycolysis increased, whereas the AMP:adenosine triphosphate (ATP) and phosphocreatine/creatine (PCr:Cr) ratios, and AMPK activity remained unchanged. Wortmannin, the well-known phosphatidylinositol-3-kinase inhibitor, blocked the activation of protein kinase B and the increase in glycolysis and Fru-2,6-P_2 content induced by increased work. Therefore, the control of heart glycolysis by contraction differs from that in skeletal muscle where AMPK is involved.
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Key words: Adenosine monophosphate-activated protein kinase; Heart work; Fructose-2,6-bisphosphate; Protein kinase B; Wortmannin

1. Introduction

Increasing cardiac work stimulates glycolysis to cope with the extra energy demand [1]. This adaptive response of the heart involves an increase in glucose uptake and fructose-2,6-bisphosphate (Fru-2,6-P_2) content [2]. Fru-2,6-P_2 is synthesized by 6-phosphofructo-2-kinase (PFK-2) and stimulates 6-phosphofructo-1-kinase (PFK-1), a key enzyme of glycolysis. Myocardial ischemia also stimulates glycolysis. The latter is mediated by adenosine monophosphate (AMP)-activated protein kinase (AMPK), which senses the energy charge of the cell and is activated by an increase in the AMP:adenosine triphosphate (ATP) ratio [3,4]. AMPK activates heart PFK-2 and so increases Fru-2,6-P_2 concentration [3], and it stimulates glucose transport through the translocation of GLUT-4 to the plasma membrane [5]. In skeletal muscle, AMPK activation is responsible for the stimulation of glycolysis which results from either oxygen deprivation or muscle contraction, both of which increase the AMP:ATP ratio [6,7]. In this tissue, the extent of AMPK activation depends on the force of contraction [8]. Therefore, AMPK involvement in the control of glycolysis has been postulated to occur in hearts submitted to increased workload [9]. Here, we tested this hypothesis and we investigated the role of AMPK in the stimulation of glycolysis and more particularly in the activation of PFK-2 by increased heart work in the isolated working heart. In this model, contraction and the work produced by the heart were modulated by changing the afterload. We showed that, in contrast to skeletal muscle, AMPK was not activated by increased heart work and that PFK-2 activation was prevented by wortmannin, a well-known inhibitor of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (PKB/Akt) axis.

2. Materials and methods

Hearts of fed male Wistar rats (250–300 g, anesthetized with 60 mg/kg pentobarbital intraperitoneally) were first perfused retrogradely via the aorta for 5 min at 75 cm H_2O, while the left atrium was cannulated. The hearts were then perfused in 'working' conditions as in [10], at 37°C in a recirculating system with 100 ml of a Krebs–Henseleit bicarbonate buffer containing 5 mM glucose and 2.5 mM $CaCl_2$, if not otherwise stated, and in equilibrium with a gas phase containing 95% O_2 and 5% CO_2, except under hypoxic (95% dry air and 5% CO_2) or anoxic (95% N_2 and 5% CO_2) conditions. The perfusion protocols are described in the legends to the figures and table. Hearts were freeze-clamped at the indicated times for measurement of metabolites or enzyme activities. The 'low load' and 'high load' conditions corresponded to hearts perfused with an afterload of 60 and 120 cm H_2O respectively, the preload being 20 cm H_2O in all conditions. In a second set of experiments, hearts were perfused at 100 cm H_2O and then submitted to an acute increase in afterload up to 140 cm H_2O. Measurements of heart rate, peak systolic pressure, mean aortic blood pressure (MABP), and aortic and coronary outputs and calculation of heart work were as in [2]. Samples of the frozen left ventricules were homogenized at 4°C in 9 Vol of a lysis buffer [3]. AMPK [3], PKB (immunoprecipitated with anti-PKBα from UBI) [11] and PFK-2 activity [3], Fru-2,6-P_2 [12], AMP, adenosine diphosphate (ADP), ATP [13] and phosphocreatine (PCr) and creatine (Cr) content [14] were measured as described. Acetyl-CoA carboxylase (ACC) phosphoryla-

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Abbreviations: ACC, acetyl coenzyme A carboxylase; AMPK, adenosine monophosphate (AMP)-activated protein kinase; Cr, creatine; p70S6K, p70 ribosomal S6 kinase; PI3K, phosphatidylinositol-3-kinase; PCr, phosphocreatine; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; PKB, protein kinase B

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Table 1
Hemodynamic parameters of working hearts submitted to an acute increase in afterload

		Heart rate (min^{-1})	MABP (mm Hg)	Coronary output (ml/min)	Cardiac output (ml/min)	Hydraulic power (mW)
Without DMSO	60 cm H_2O	236 ± 10	51 ± 1	8.6 ± 0.8	28.4 ± 5.4	3.1 ± 0.6
	60 → 120 cm H_2O, 1 min	N.D.	99 ± 2*	23.4 ± 1.8*	45.8 ± 5.7*	9.8 ± 1.3*
	60 → 120 cm H_2O, 15 min	237 ± 25	100 ± 1*	21.8 ± 2.6*	40.3 ± 4.2*	8.7 ± 0.9*
DMSO	60 cm H_2O	235 ± 27	54 ± 2	8.9 ± 0.8	32.0 ± 6.3	3.8 ± 0.8
	60 → 120 cm H_2O, 15 min	246 ± 31	99 ± 2*	23.6 ± 0.7*	40.9 ± 4.2*	8.8 ± 0.8*
WMN	60 cm H_2O	218 ± 17	53 ± 2	9.7 ± 0.5	29.4 ± 4.7	3.5 ± 1.3
	60 → 120 cm H_2O, 15 min	227 ± 31	93 ± 2*	21.6 ± 0.9*	31.8 ± 4.2	6.2 ± 0.8*

Hearts were submitted to an acute increase in afterload (120 cm H_2O) after a 15 min period of equilibration at 60 cm H_2O. The hemodynamic parameters were measured after 1 and 15 min of increased workload as indicated. Wortmannin (WMN), dissolved in dimethylsulfoxide (DMSO), was added to the perfusion medium after 5 min of equilibration to reach a final concentration of 100 nM. The hearts were then submitted to an increase in afterload (from 60 cm H_2O to 120 cm H_2O) after 15 min of equilibration. The hemodynamic parameters were measured after 15 min of increased workload as indicated. DMSO was the vehicle. The values are the means ± S.E.M. of at least six hearts. *$P < 0.01$ indicates values that are statistically different from the control hearts. N.D., non-determined.

tion was evaluated by immunoblotting with anti-phosphoSer-79ACC (generous gift from D.G. Hardie, Dundee), which recognizes the cognate Ser-227 in ACC2 [15]. Glycolysis was estimated by the rate of detritiation of [3-^{3}H]glucose [16]. Proteins were measured by Coomassie blue staining with bovine serum albumin as a standard. Except otherwise stated, the values are means ± S.E.M., the statistical significance of differences was calculated by the Student's *t*-test.

3. Results

3.1. Increased heart work stimulates glycolysis without change in AMPK activity

Hearts were perfused at 'low load' (60 cm H_2O) and then submitted to an acute increase in afterload ('high load', 120

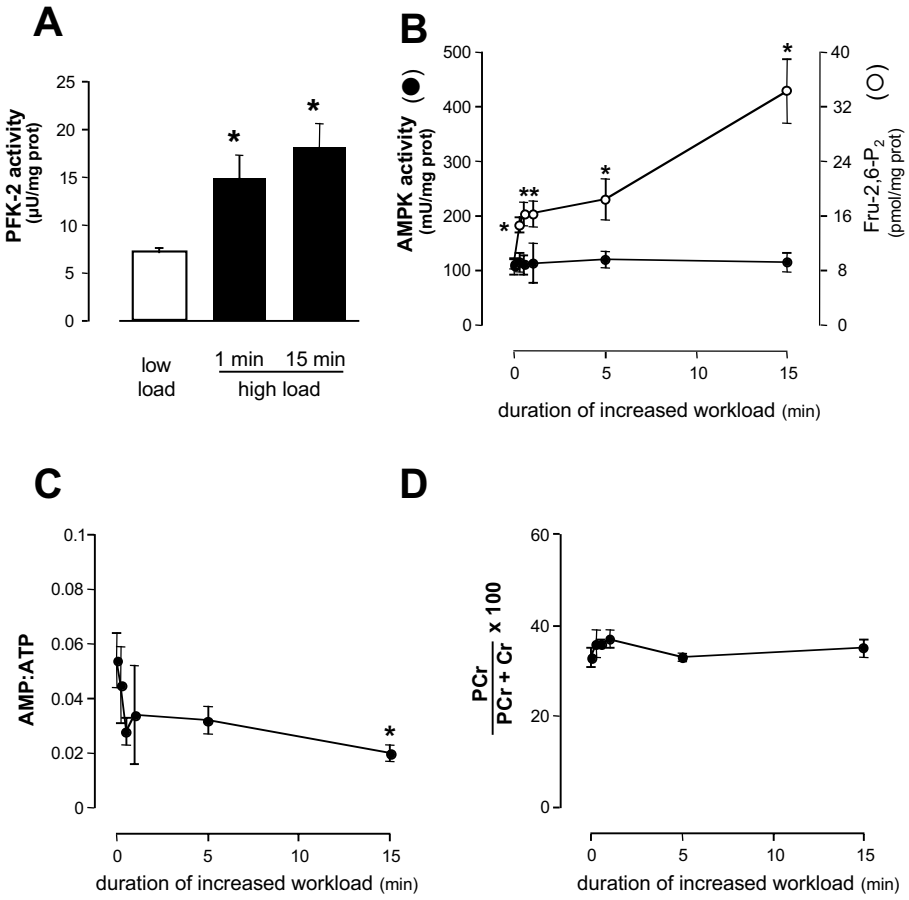

Fig. 1. Effect of acute increase in pressure load on PFK-2 (A) and AMPK (B) activity, Fru-2,6-P_2 content (B), AMP/ATP (C) and PCr/Cr ratio (D). Hearts were submitted to an acute increase in afterload (120 cm H_2O) after a 15 min period of equilibration at 60 cm H_2O. The hearts were freeze-clamped at the indicated times for measurement of AMPK and PFK-2 activity, and Fru-2,6-P_2, AMP, ATP, PCr and Cr content. The ATP concentration was 2.66 ± 0.15 and 2.71 ± 0.17 µmol/g tissue, the PCr concentration was 3.15 ± 0.22 and 3.19 ± 0.2 µmol/g tissue, at 'low load' and at 15 min 'high load', respectively. The values are the means ± S.E.M. for at least five hearts. *$P < 0.05$ indicates values that are statistically different from control hearts.

cm H_2O). The acute change in load increased the work (Table 1) as well as PFK-2 activity and Fru-2,6-P_2 concentration (Fig. 1A, B). It also increased the flux through PFK-1 measured by the rate of detritiation of [3-^{3}H]glucose ('low load', 36 ± 4; 'high load', 53 ± 4 μmol glucose h^{-1} g tissue^{-1}, $n = 3$, $P < 0.05$). In contrast, AMPK remained remarkably constant during this transition from 'low to high load' (Fig. 1B). Allosteric effectors of AMPK [17] were also measured in these hearts. No change in Cr, PCr and ATP were detected, whereas a small decrease in AMP, and hence in the AMP:ATP ratio, was detected at 1 min which became statistically significant at 15 min (Fig. 1C, D). These results rule out any activation or allosteric stimulation of AMPK under these conditions.

In another series of experiments, hearts were equilibrated at 100 cm H_2O for 15 min and then submitted to an acute increase in afterload of 140 cm H_2O for up to 10 min to mimic a pressure overload. Again, this experimental protocol induced an increase in Fru-2,6-P_2 content (control, 28.7 ± 2.6; 140 cm H_2O, 10 min, 38.1 ± 2.8 pmol/mg protein, $n = 4$, $P < 0.05$) but failed to activate AMPK at any time points (control, 109 ± 20; 140 cm H_2O, 1 min, 74 ± 13; 140 cm H_2O, 10 min, 118 ± 20 mU/mg protein, $n = 3$, P is not significant). Increasing Ca^{2+} concentration from 1.5 to 10 mM in the perfusion medium of hearts equilibrated at 100 cm H_2O, which is known to increase the force of contraction [18], did not change AMPK activity (data not shown).

Comparison of the effects of oxygen restriction with those of increased afterload showed that the increase in Fru-2,6-P_2 content was similar in both conditions, whereas AMPK was only activated in hypoxia or anoxia (Fig. 2A). Moreover, Ser-227 phosphorylation in ACC2, a known substrate of AMPK [19], increased only when the oxygen supply was restricted (Fig. 2B). In conclusion, AMPK activation was not observed when heart work was increased, whatever the time-course, the mean aortic blood pressure or the Ca^{2+} concentration used in the perfusate.

3.2. *Wortmannin inhibits PFK-2 and PKB activation by increased workload*

Perfusing hearts with wortmannin, the well-known inhibitor of PI3K, prevented both the increase in Fru-2,6-P_2 content and the stimulation of glycolysis resulting from the increased workload (Fig. 3A and B). Wortmannin also decreased cardiac output without changing the mean aortic blood pressure and coronary flow (Table 1); it had no effect on AMPK activity (data not shown). Therefore, PI3K appears to be involved in the hemodynamic and metabolic adaptation of the heart to an acute increase in pressure load.

PKB is activated by insulin in the heart in a wortmannin-sensitive manner [20] and it is able to phosphorylate and activate heart PFK-2 in vitro [21]. Perfusing hearts at high load resulted in PKB activation, which was prevented by wortmannin (Fig. 3C). p70 ribosomal S6 kinase (p70S6K) is another protein kinase that is activated by insulin in the heart by a wortmannin-sensitive mechanism [22], and that is able to phosphorylate and activate heart PFK-2 in vitro [21]. p70S6K activation involves several protein kinases, one of which is the mammalian target of rapamycin, which is inhibited by rapamycin [23]. A role for p70S6K in PFK-2 activation was ruled out since, by contrast with PKB, p70S6K activity remained unchanged when the workload was increased and since rapamycin had no effect on Fru-2,6-P_2 content after the increase in pressure load (data not shown).

4. Discussion

Contraction leads to AMPK activation in isolated muscle preparations as well as in vivo [24]. Our data demonstrate that this does not hold true for the heart. Indeed increasing heart work stimulated glycolysis but did not activate AMPK or change the AMP:ATP or PCr:Cr ratios. However, AMPK was activated after oxygen deprivation, indicating that AMPK activation is restricted to stress conditions and that the normoxic heart adapts its metabolism to the extra energy demand without involving AMPK.

Both oxygen deprivation and increasing workload stimulate

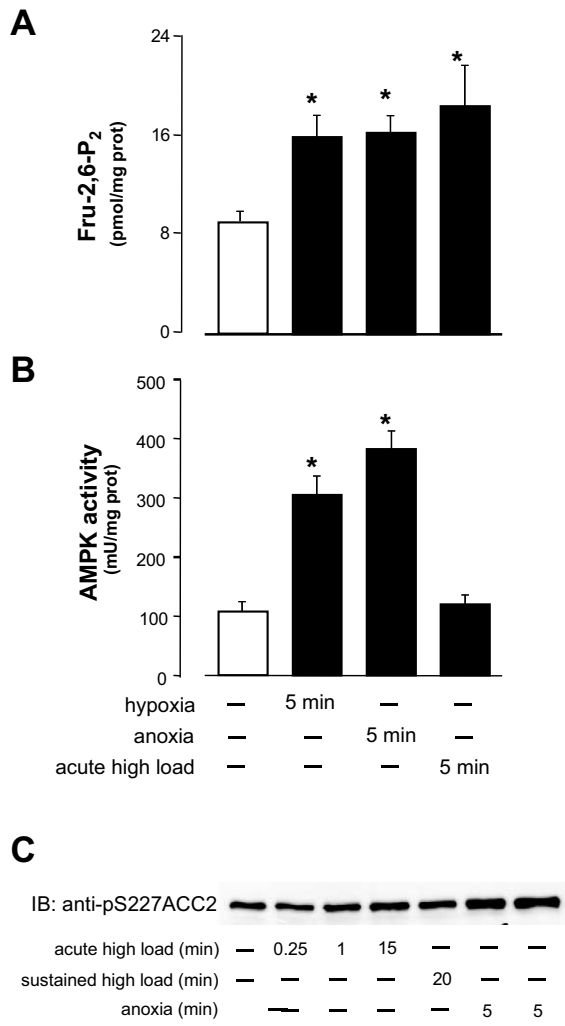

Fig. 2. Effect of hypoxia, anoxia and workload on Fru-2,6-P_2 content (A), AMPK activity (B), and ACC phosphorylation state (C). The hearts were perfused for 15 min at 60 cm H_2O and then submitted to hypoxia or anoxia for 5 min. They were compared with hearts submitted to an increase in afterload as in Fig. 1. The values are the means ± S.E.M. for at least five hearts. *$P < 0.05$ indicates values that are statistically different from control hearts. C: Phosphorylation state of Ser-227 in ACC2; 50 μg of protein from heart extracts were loaded on each lane for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The blot shown is representative of three experiments.

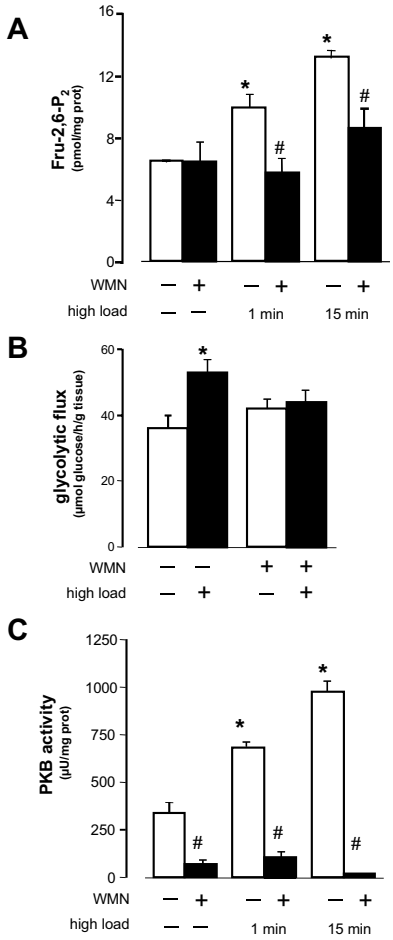

Fig. 3. Effect of wortmannin on Fru-2,6-P_2 content (A), glycolytic flux (B) and PKB activity (C) in hearts submitted to an acute increase in afterload. Wortmannin (WMN) was added to the perfusion medium after 5 min of equilibration to reach a final concentration of 100 nM. The hearts were submitted to an increase in afterload (from 60 cm H_2O to 120 cm H_2O) after 15 min of equilibration and freeze-clamped at 1 and 15 min under high load condition to measure Fru-2,6-P_2 content (A) and PKB activity (C). The values are the means ± S.E.M. for at least four hearts. $*P < 0.05$ indicates values that are statistically different from the low load hearts, equilibrated at 60 cm H_2O. $\#P < 0.05$ indicates values that are statistically different from the corresponding untreated hearts.

heart glycolysis by enhancing glucose transport and by increasing PFK-2 activity. However, the mechanisms involved are different. AMPK mediates the effects of anoxia. Our present results show that PFK-2 activation by workload, like the metabolic effects of insulin, are wortmannin-sensitive. The wortmannin-sensitive stimulation of cardiac glucose transport during contraction has already been described in a model of electrically stimulated cardiomyocytes [25]. In this model, the stimulation of glucose transport could not be related to changes in activity or phosphorylation of the insulin receptor, the insulin receptor substrate-1 or insulin receptor substrate-1 associated PI3K, indicating that the upstream signalling differs from that of insulin. In addition, the authors failed to demonstrate any activation of protein kinases downstream of PI3K that could be involved in the stimulation of glucose transport. In our model of isolated working heart, we showed that PKB was activated by workload to an extent that is similar to that observed in hearts perfused with 1 nM insulin [11]. Therefore, PKB appears as a possible candidate to mediate PFK-2 activation.

Wortmannin did not inhibit basal PFK-2 activity, as already reported [26], although it inhibited PKB. This suggests that PFK-2 was not activated when hearts were perfused at 'low load' (subphysiological conditions). Indeed, PFK-2 activity as well as Fru-2,6-P_2 content were close to those found in non-working hearts in which no PFK-2 phosphorylation was detected [3].

Our data do not allow us to exclude the involvement of protein kinases other than PKB, downstream of PI3K, in PFK-2 activation by workload. Activation of the PI3K/PKB axis has been reported to occur in contracting skeletal muscle [27] as well as in endothelial cells submitted to laminar shear stress [28]. This suggests that mechanical forces could play a role in the activation of the PI3K/PKB pathway in different tissues. The mechano-transducer and the signalling pathway linking this transducer to PI3K remain to be elucidated.

Wortmannin limits the work developed after an increase in afterload. This could result from a decrease in energy supply, as the workload-induced stimulation of glycolysis was inhibited. Similarly, wortmannin treatment counteracts the positive inotropic effect of insulin in the post-ischemic heart [29]. However, the data do not allow one to exclude the possibility that the activation of the PI3K/PKB pathway improves heart function, independent of its metabolic effects, by controlling either excitation–contraction coupling and/or muscle contraction. The latter is supported by a recent work showing that PI3K influences contraction of vascular smooth muscle cells [30].

Our results suggest that the PI3K/PKB pathway could participate in the adaptation of cardiac function to an increased afterload. Moreover, PKB could mediate other effects. Indeed, overexpression of PKB induces a moderate cardiac hypertrophy with preserved systolic function [31]. Thus, it is not excluded that, in addition to its metabolic effects, the PI3K/PKB pathway could participate in the early events leading to the adjustment of cardiac mass to hemodynamic load. In contrast, AMPK appears not to be involved in the genesis of hypertrophy in response to acute pressure load as it has been already suggested [32].

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