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# ATP depletion increases phosphorylation of elongation factor eEF2 in adult cardiomyocytes independently of inhibition of mTOR signalling

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**Abstract Translation elongation consumes a high proportion of cellular energy and can be regulated by phosphorylation of elongation factor eEF2 which inhibits its activity. We have studied the effects of ATP depletion on the phosphorylation of eEF2 in adult rat ventricular cardiomyocytes. Energy depletion rapidly leads to inhibition of protein synthesis and increased phosphorylation of eEF2. Stimulation of the AMP-activated protein kinase also causes increases eEF2 phosphorylation. Only at later times is an effect on mTOR signalling observed. These data suggest that energy depletion leads to inhibition of protein synthesis through phosphorylation of eEF2 independently of inhibition of mTOR signalling.**


*Key words:* Protein synthesis; Elongation; mTOR; ATP; AMP-activated protein kinase; Cardiomyocyte

## 1. Introduction

Protein synthesis consumes a high proportion of cellular energy (typically 25–30% [1]). In particular, the elongation process consumes at least four high-energy phosphodiester bonds for each amino acid residue added to the growing chain. Cells have devised mechanisms to match the availability of metabolic energy to the demand for energy by anabolic processes and its availability, e.g. from fuel oxidation. One important example of this is the 5′AMP-activated protein kinase (AMPK) which is activated when the cellular energy charge falls due to a rise in the AMP/ATP ratio [2]. AMPK is known to phosphorylate a number of enzymes and other proteins to shut off energy-requiring processes and favour those that can generate metabolic energy. It has also been suggested that reduced cellular ATP levels decrease the activity of the protein mTOR (mammalian target of rapamycin; mTOR activity is inhibited by rapamycin; [3]), which lies upstream of, and regulates, several proteins involved in mRNA translation.

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*Abbreviations:* AICAR, 5-aminoimidazole-4-carboxyamide 1-β-D-ribofuranoside; AMPK, 5′AMP-activated protein kinase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; eEF2, eukaryotic elongation factor 2; eIF, eukaryotic initiation factor; mTOR, mammalian target of rapamycin; ZMP, 5-aminoimidazole-4-carbonamide riboside monophosphate

These targets for mTOR signalling include the kinases that phosphorylate ribosomal S6 [4]. Two S6 kinase genes exist in mammals, the product of the S6K1 gene being better understood than S6K2. Rapamycin blocks the activation of the S6 kinases, indicating an essential role for mTOR in their regulation. mTOR is also required for regulation of the eukaryotic initiation factor (eIF) 4E-binding protein, 4E-BP1, which in its hypophosphorylated state binds to and inhibits eIF4E. eIF4E interacts with the 5′-cap of the mRNA (which contains 7-methylGTP) and also binds the scaffold protein eIF4G, thereby recruiting other factors and the 40S ribosomal subunit to the mRNA [5]. Insulin induces the phosphorylation of 4E-BP1 and its release from eIF4E to facilitate cap-dependent translation [5,6].

The translation elongation process is regulated through the phosphorylation of eukaryotic elongation factor (eEF) 2. Phosphorylation at Thr56 inactivates eukaryotic elongation factor 2 (eEF2) and is catalysed by a very specific and unusual protein kinase, eEF2 kinase [7,8] (formerly termed $Ca^{2+}$/calmodulin kinase III [9,10]). Insulin elicits dephosphorylation of eEF2 by inactivating eEF2 kinase [11,12], and this involves phosphorylation of eEF2 kinase by S6K1 at Ser365 (rat sequence [13]). Inactivation of eEF2 kinase and the dephosphorylation of eEF2 in response to insulin are sensitive to rapamycin, reflecting this input from S6K1 [11].

Heart muscle cells have a high and varying requirement for metabolic energy, the demand increasing as contraction is stimulated. We have previously shown that β-adrenergic stimulation, which exerts positive inotropic and chronotropic effects on contraction, causes activation of eEF2 kinase and phosphorylation of eEF2 in isolated adult rat cardiomyocytes (ARVC [14]). This presumably serves to reduce the rate of protein synthesis to divert energy to support contraction. Heart muscle is a highly oxidative tissue with much of its energy being generated by mitochondrial oxidation of pyruvate or fatty acids. Cardiac energy supply is thus greatly impaired by interruption of the supply of oxygen (such as occurs in cardiac ischaemia or anoxia). This effect can be mimicked in vitro by inhibiting mitochondrial function. Here we have explored the effects of such manipulations on phosphorylation of eEF2 and on targets of the mTOR pathway. Our data show that increased phosphorylation of eEF2 is an immediate effect of disturbing energy metabolism in this way, and that impairment of mTOR signalling only occurs later and/or under more severe conditions. Our data also imply a role for AMPK in regulating the phosphorylation of eEF2 under conditions of ATP depletion.

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## 2. Materials and methods

### 2.1. Materials

The mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and the ATP-synthesis inhibitor oligomycin were from Sigma Chemical, Poole Dorset, UK; 5-aminoimidazole-4-carboxyamide 1-β-D-ribofuranoside (AICAR) was from Toronto Research Chemicals, ON, Canada. Rats were from Charles River (Margate, Kent, UK). The antisera for eEF2, eEF2 phosphorylated at Thr56, 4E-BP1, eIF4E, eIF4G and S6K1, have been described previously [14–19]. The antibody that recognises acetyl-CoA carboxylase when phosphorylated at Ser79 was a kind gift from Professor Grahame Hardie, Dundee (see [20]). [$^{35}$S]Methionine was from Amersham Biosciences (Little Chalfont, Bucks, UK).

### 2.2. Isolation, culture and treatment of adult rat ventricular myocytes

Adult rat ventricular cardiomyocytes were isolated and maintained exactly as described earlier [12]. Where indicated, cells were treated with insulin (10 nM, 20 min) prior to the addition of further stimuli. Extracts were prepared as described earlier using our standard lysis buffer, which contains a cocktail of protease and phosphatase inhibitors [12]. Protein concentrations were determined by Bradford's method [21].

### 2.3. Gel electrophoresis and western blotting

Gel electrophoresis was performed as described earlier [12], using running gels with the indicated percentage of acrylamide: for S6K1, 10%; for 4E-BP1, for 12.5%; for eEF2, 10%; for ACC, 8%. Transfer was by the 'wet' method. In all cases, Immobilon® membrane was used. Blots were visualised by enhanced chemiluminescence (ECL). For analysis of the binding of eIF4G and 4E-BP1 to eIF4E, proteins were subjected to affinity chromatography on m$^7$GTP-Sepharose as described in [17].

### 2.4. Other procedures

Protein synthesis was assayed exactly as described earlier [14]. For determination of adenine nucleotides, overnight cultures of ARVC were extracted in ice-cold 7% perchloric acid and centrifuged at 10 000×*g* for 10 min at 4°C. The protein pellet was resuspended in 1 M NaOH and the protein content determined by Bradford's assay [21]. The supernatant was neutralised with 3 M $KHCO_3$ to approximately pH 5. The potassium perchlorate was centrifuged out and the supernatant was frozen in liquid nitrogen. Samples were analysed in duplicate by HPLC, using a Supelcosil LC-18-T column (3-μM pore size) and an isocratic gradient of 0.1 M $KH_2PO_4$/4 mM tetrabutylammonium hydrogen sulphate (phase A) and phase A:MeOH (70:30, phase B). These analyses were kindly performed by Dr Steve Land,

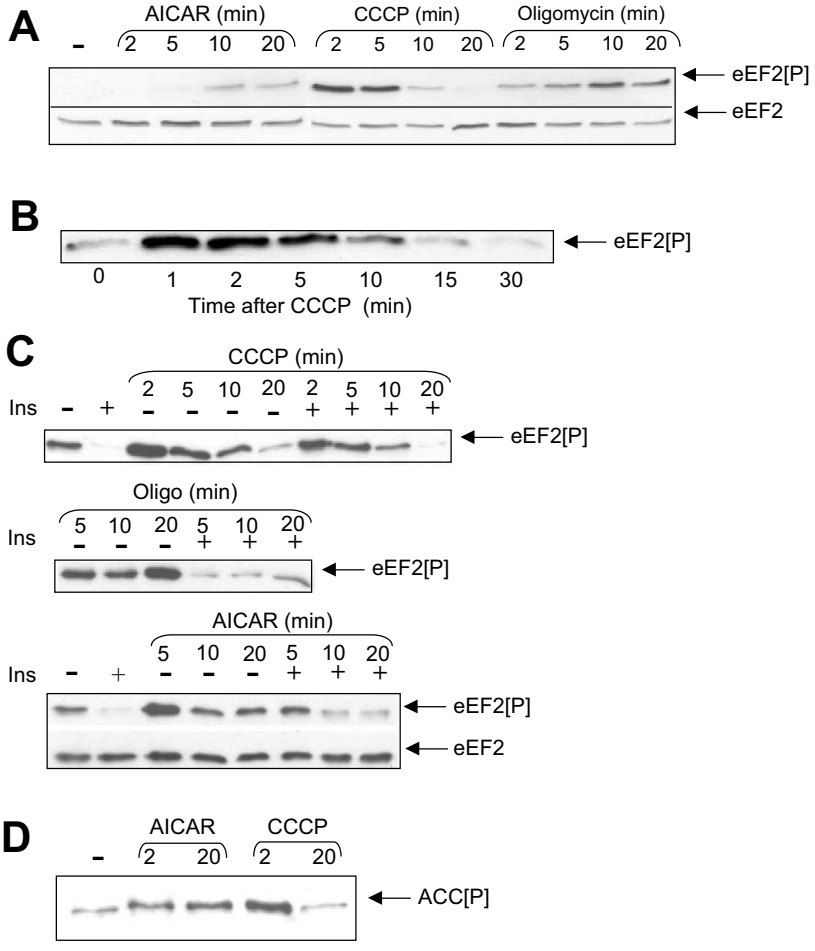


Fig. 1. ATP depletion or AICAR treatment increases phosphorylation of eEF2. A: ARVC were treated with insulin (see Materials and methods) and then with the indicated agent (AICAR, 1 mM; CCCP, 10 μM; oligomycin, 1 μM) for the times shown. Cells were then lysed and equal amounts of cell protein (usually 30 μg) were analysed by SDS–PAGE (10% gel), followed by Western blotting with an antibody that recognises eEF2 only when phosphorylated at Thr56 (upper part) or, as loading control, one that binds eEF2 irrespective of its state of phosphorylation. −, no treatment other than insulin. B: As A, but cells were not preincubated with insulin and were only treated with CCCP. Control blots confirmed equal loading (not shown). C: As A, but some dishes were not treated with insulin (−), while others were (+), prior to addition of the agents shown (concentrations as above). Equal loading was confirmed by a parallel blot using the anti-eEF2 antibody mentioned in A, but this control is only shown for AICAR-treated cells. D: As A, except cells were treated with AICAR or CCCP for the times shown, and the blot was probed with an antibody that recognises ACC when it is phosphorylated at Ser79. Similar data were obtained in four (D) or five (other panels) separate experiments.

Table 1
Effects of ATP depletion or AICAR on protein synthesis in ARVC

| Condition | Rate of protein synthesis ([$^{35}$S]methionine incorporation as % of insulin-treated control) | *n* |
|---|---|---|
| AICAR, 1 mM | | |
| 5 min | 69.5 ± 1.4 | 6 |
| 20 min | 66.3 ± 5.0 | 7 |
| CCCP, 10 μM | | |
| 2 min | 4.8 ± 2.9 | 9 |
| 5 min | 7.9 ± 1.7 | 8 |
| 10 min | 11.1 ± 6.8 | 9 |
| Oligomycin, 1 μM | | |
| 5 min | 38.8 ± 6.9 | 9 |
| 20 min | 43.0 ± 2.1 | 6 |

ARVC were preincubated with insulin for 20 min and then treated as indicated. [$^{35}$S]Methionine was added immediately after the time indicated for a period of 15 min, after which cells were lysed and samples processed for measurements of incorporation of label into protein [14] and protein concentration [21]. Data are expressed as % of insulin-treated (mean ± S.E.M. (*n* is indicated for each set)) and are normalised for the protein content of the lysate.

Tayside Institute of Child Health, Ninewells Hospital, Dundee, as described in [20].

## 3. Results and discussion

### 3.1. ATP depletion rapidly leads to inhibition of protein synthesis and phosphorylation of eEF2

Treatment of ARVC with the uncoupler CCCP resulted in a very marked inhibition of protein synthesis within 2 min (Table 1; it is not possible to study protein synthesis over shorter time intervals). Oligomycin treatment also markedly inhibited protein synthesis (Table 1).

Given the substantial inhibition of protein synthesis seen here, it was important to examine whether CCCP treatment affected the phosphorylation of proteins that are known to regulate overall protein synthesis rates. We analysed eEF2 and eIF2α because phosphorylation of either can cause a general inhibition of mRNA translation [7,22]. As shown in Fig. 1A, CCCP rapidly and markedly increased the phosphorylation of eEF2 at its regulatory site, Thr56. Phosphorylation was maximal by 1 min after addition of CCCP (Fig. 1B). At later times, eEF2 phosphorylation fell. We surmise that, at these later times, cellular ATP levels are so low that phosphorylation of eEF2 by eEF2 kinase within the cells is impaired.

Treatment of ARVC with oligomycin (which inhibits mitochondrial ATP synthesis, rather than dissipating the electrochemical gradient across the mitochondrial membrane), also caused increased phosphorylation of eEF2, although the effect was slower in onset. Even by 10 or 20 min of treatment, the level of eEF2 phosphorylation was still lower than that observed after 2 min of treatment with CCCP (Fig. 1A).

Insulin decreases the level of phosphorylation of eEF2 in ARVC, as confirmed here (Fig. 1C) [12]. Following treatment of ARVC with insulin, CCCP or oligomycin increased the phosphorylation of eEF2, although not to the levels seen in the absence of insulin (Fig. 1C). The effects of insulin and ATP-depleting agents on the phosphorylation of eEF2 therefore appear to be exerted through mutually antagonistic mechanisms.

### 3.2. AICAR, an activator of AMPK, also causes phosphorylation of eEF2

Treatment of cells with CCCP (which collapses the electrochemical gradient across the mitochondrial membrane) is expected to have a marked effect on the adenylate energy charge within the cell. Measurements of the concentrations of ATP, ADP and AMP revealed that in control cells the energy charge (defined as ATP+1/2ADP/[total adenine nucleotides], [23]) was 0.79, in line with earlier findings for cardiomyocytes [24] (Table 2). As anticipated, treatment with CCCP rapidly decreased the ratio, with a substantial change already being evident by 2 min.

One mechanism by which a fall in the cellular energy charge (i.e. an increased AMP/ATP ratio) could affect the phosphorylation of eEF2 is through activation of AMPK. Targets for AMPK include acetyl-CoA carboxylase (ACC), an enzyme which is important in regulating both anabolic and catabolic processes. To assess whether the conditions used here led to activation of AMPK, we examined the phosphorylation of Ser79 in ACC. Treatment of ARVC with CCCP caused a marked and rapid rise in the phosphorylation of ACC (Fig. 1D), indicative of a rise in the activity of AMPK by 2 min, a time at which phosphorylation of eEF2 is maximal (Fig. 1A,B). By 20 min, phosphorylation of ACC had declined. This is similar to the situation for eEF2 (Fig. 1A,B) and may arise for similar reasons.

The data above suggested that AMPK might be involved in regulating the phosphorylation of eEF2 in ARVC. To test this, we treated ARVC with AICAR, a compound that, upon intracellular conversion to 5-aminoimidazole-4-carbonamide riboside monophosphate (ZMP), yields an activator of AMPK [2]. It has previously been reported that AICAR is rather ineffective in activating AMPK in heart cells, probably due to inefficient conversion to ZMP [25]. Nonetheless, we did see a modest increase in the phosphorylation of eEF2 upon treatment of our adult cardiomyocytes with AICAR, both in

Table 2
Adenylate energy charge of ARVC

| Condition | Energy charge |
|---|---|
| Control | 0.79 ± 0.01 |
| AICAR, 5 min | 0.79 ± 0.02 |
| AICAR, 10 min | 0.75 ± 0.02 |
| AICAR, 15 min | 0.78 ± 0.02 |
| CCCP, 2 min | 0.15 ± 0.07 |
| CCCP, 5 min | 0.11 ± 0.03 |
| CCCP, 10 min | 0.09 ± 0.03 |

ARVC were treated as indicated (CCCP, 10 μM; AICAR, 1 mM) for the times shown and then acid extracts were prepared and processed for measurement of the levels of adenine nucleotides. The adenylate energy charge is defined as [ATP]+1/2[ADP]/total (ATP +ADP+AMP). Data are given as mean ± S.E.M. (*n* = 4).

the absence and presence of pretreatment of the cells with insulin (Fig. 1A,C). As expected, AICAR did not alter the adenylate charge within the cells (Table 2). It was clear from the HPLC analysis of the nucleotide levels that treatment of cells with AICAR led to the accumulation of a peak corresponding to ZMP, which was not observed for cells that had not been treated with AICAR. Based on the mean of duplicate determinations (which gave results within 10% of one another), levels of ZMP were 0.02, 0.09 and 0.22 nmol/mg protein at times of 5, 10 and 20 min after addition of AICAR to the cells. These are similar to the concentrations measured in skeletal muscles of rats injected with AICAR, and which activated AMPK [26]. Treatment of ARVC with AICAR also caused an increase in the phosphorylation of ACC, although not to the extent seen in response to CCCP (Fig. 1D). These data suggest that, consistent with recent findings for certain other cell types [27], activation of AMPK leads to increased phosphorylation of eEF2. The mechanisms underlying this remain unclear [27], and are currently under investigation using more tractable cell types than ARVC. AICAR treatment also inhibits protein synthesis in ARVC (Table 1), although to a smaller effect than CCCP. This may reflect the fact that AICAR increases eEF2 phosphorylation to a lesser extent than CCCP (Fig. 1A,C).

Analysis of the phosphorylation of eIF2α failed to reveal any change in the phosphorylation of this protein in response to CCCP, oligomycin or AICAR in several separate experiments (data not shown). Thus, inhibition of protein synthesis caused by mitochondrial poisons correlates with phosphorylation of eEF2 (an event known to impair overall protein synthesis) but is not accompanied by any change in the phosphorylation of another regulator of total protein synthesis, eIF2. This differs from the conclusions of Munoz et al. [28], who, using a quite different cell type (PC12 cells), found that CCCP treatment did cause an increase in the phosphorylation of eIF2α. This effect was not observed in our study, indicating that this is not a universal response of mammalian cells to ATP depletion.

### 3.3. *Only prolonged treatment with CCCP affects targets of mTOR signalling*

Recent work has shown that the activity of eEF2 kinase can be decreased following its phosphorylation (at Ser365 in the rat protein) by S6K1 or $p90^{RSK}$ [13]. We therefore considered the possibility that depletion of ATP might inactivate these upstream kinases, thereby increasing eEF2 kinase activity and also eEF2 phosphorylation. In the case of $p90^{RSK}$, its upstream activators, Erk1/2 are essentially inactive either in untreated ARVC or after insulin treatment [12]. Consonant with this, $p90^{RSK}$ is inactive in such cells (L. Wang, unpublished data) and a role for inactivation of $p90^{RSK}$ can therefore be rejected. S6K1 does show basal activity in ARVC and its phosphorylation/activity are increased by insulin [12]. It was therefore possible that ATP-depletion led to phosphorylation of eEF2 by causing the dephosphorylation and inactivation of S6K1. Phosphorylation of S6K1 (which is linked to its activation) is associated with a retardation of its mobility on SDS–PAGE. In insulin-treated cells, S6K1 appeared as three distinct bands on SDS–PAGE, the middle one being the strongest. Treatment of ARVC with CCCP for 1 min, a time at which eEF2 phosphorylation is already maximal, had no effect on the mobility of S6K1 (Fig. 2, compare first and last lanes). At later times, a shift to faster migrating, less phosphorylated forms was apparent. An increase in the intensity of the fastest migrating band (starred in Fig. 2) was observed by 2 min and this became the main species by 10 min. This indicates inactivation of S6K1. Oligomycin treatment also resulted in dephosphorylation of S6K1, but the effect was smaller and slower, with S6K1 migrating in roughly equal proportions of the middle and fastest species by 20 min.

The data are consistent with the idea proposed by Dennis et al. [3] that ATP depletion causes inactivation of signalling through mTOR. However, this change is markedly slower in onset than the increase in eEF2 phosphorylation (which occurs by 1 min of CCCP treatment, Fig. 1), possibly because severe or prolonged ATP depletion is required for this effect (in agreement with the data of [27]). To study this further we examined a second target of mTOR signalling, the translational repressor 4E-BP1.

In its hypophosphorylated state, 4E-BP1 binds to and inhibits eIF4E. In response to, e.g. insulin, 4E-BP1 undergoes phosphorylation at multiple sites and is released from eIF4E, e.g. in ARVC [12]. ARVC were treated with insulin for 20 min and then with CCCP for different times before being lysed. Lysates were then subjected to affinity chromatography on $m^7$GTP-Sepharose, which binds eIF4E and associated proteins. The bound material was analysed by SDS–PAGE and Western blotting. Since this analysis requires very large amounts of material (approx. 800 μg, equivalent to 40% of the cells from one rat heart), it is only possible to study a limited range of conditions. No detectable 4E-BP1 was associated with eIF4E in insulin-treated ARVC, as reported earlier [12] (Fig. 3). Treatment with CCCP caused an increase in 4E-BP1 binding by 5 min, but this was only partial, and binding increased further by 15 and 30 min of treatment. Indeed, a substantial amount of eIF4G was still clearly associated with eIF4E at 5 min, although this was lost (due to increased binding of eIF4E to 4E-BP1) by later times. The increased binding of 4E-BP1 to eIF4E, which indicates inhibition of mTOR signalling, occurs later than the much more rapid rise in eEF2 phosphorylation (Fig. 1). To study earlier times, we treated ARVC with CCCP for 2 min, a time at which eEF2

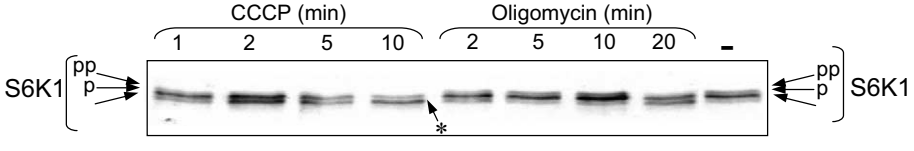


Fig. 2. ATP depletion results in dephosphorylation of S6K1. ARVC were pre-treated with insulin and then with the agents shown (CCCP, 10 μM; oligomycin, 1 μM) for the indicated times. −, cells treated only with insulin. Equal amounts of cell protein (70 μg) were analysed by SDS–PAGE (10% gel, run until the 66-kDa marker was almost at the bottom to maximise resolution) and Western blotting using anti-S6K1 antiserum. Three bands are visible, corresponding to different phosphorylated forms of the enzyme (as denoted by the labelled arrows). The starred arrow indicates the least phosphorylated form of S6K1 generated after CCCP or oligomycin treatment.

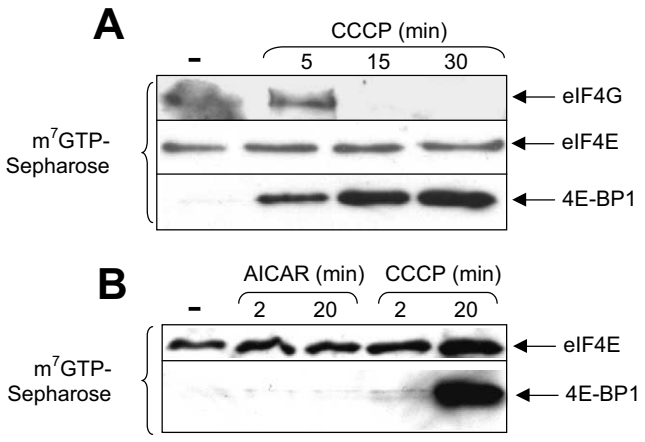


Fig. 3. CCCP causes increased binding of the translational repressor 4E-BP1 to eIF4E. A,B: ARVC were treated with insulin and then with the agents shown (CCCP, 10 μM; AICAR, 1 mM). After lysis, samples of lysate (750–800 μg protein) were subjected to affinity chromatography on m$^7$GTP-Sepharose, which binds eIF4E and proteins associated with it. Bound material was subjected to SDS–PAGE (12.5% gel) and Western blotting using antisera to eIF4E, eIF4G or 4E-BP1, as indicated. The signal for eIF4E serves as loading control to which other signals can be normalised. Similar data were obtained in four separate experiments.

phosphorylation is already maximal (Fig. 1). It is clear from Fig. 3B that there is essentially no effect of CCCP on binding of 4E-BP1 to eIF4E at this time, indicating that there is no significant impairment of signalling via the mTOR pathway. This is entirely consistent with the data shown in Fig. 2 for S6K1, which also only underwent significant dephosphorylation at relatively late times. The observation that eEF2 phosphorylation is already maximally elevated at 1 min after addition of CCCP, while S6K1 only undergoes phosphorylation at later times, strongly suggests that the changes in eEF2 phosphorylation are not due to inactivation of mTOR signalling, which can regulate eEF2 in ARVC [12], but rather to other consequences of ATP depletion. Bolster et al. [29] have recently reported that injection of AICAR into rats causes inhibition of mTOR signalling in skeletal muscle. However, activation of AMPK may also interfere with the synthesis and/or release of insulin [30,31], making it hard to interpret these data. The effects on muscle mTOR signalling may, for example, reflect changes in circulating insulin levels.

### 3.4. Conclusions

The data presented here shows that ATP depletion increases the phosphorylation of eEF2. Like the inhibition of protein synthesis, this effect is rapid. CCCP has a greater effect on protein synthesis and on eEF2 phosphorylation than oligomycin. This data suggest that phosphorylation of eEF2 plays an important role in the inhibition of protein synthesis when ATP levels fall. eEF2 phosphorylation rises before inhibition of mTOR signalling is apparent, suggesting that a mechanism other than changes in mTOR signalling is involved. The observation that AICAR induces eEF2 phosphorylation points to a key role for AMPK in these effects. Further work is required to establish how AMPK regulates eEF2 phosphorylation. This mechanism for acutely inhibiting the consumption of energy by protein synthesis could be of particular importance in assuring continued supplies of metabolic energy for contraction and ion pumps in the heart during periods of relative energy insufficiency.

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## References

[1] Schmidt, E.V. (1999) Oncogene 18, 2988–2996.
[2] Hardie, D.G., Carling, D. and Carlson, M. (1998) Annu. Rev. Biochem. 67, 821–855.
[3] Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C. and Thomas, G. (2001) Science 294, 1102–1105.
[4] Avruch, J., Belham, C., Weng, Q., Hara, K. and Yonezawa, K. (2001) Prog. Mol. Subcell. Biol. 26, 115–154.
[5] Gingras, A.-C., Raught, B. and Sonenberg, N. (1999) Annu. Rev. Biochem. 68, 913–963.
[6] Lawrence, J.C. and Abraham, R.T. (1997) Trends Biochem. Sci. 22, 345–349.
[7] Proud, C.G. (2000) in: Translational Control of Gene Expression (Sonenberg, N., Hershey, J.W.B. and Mathews, M.B., Eds.), pp. 719–39, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[8] Ryazanov, A.G. (2002) FEBS Lett. 514, 26–29.
[9] Ryazanov, A.G., Natapov, P.G., Shestakova, E.A., Severin, F.F. and Spirin, A.S. (1988) Biochimie 70, 619–626.
[10] Nairn, A.C. and Palfrey, H.C. (1987) J. Biol. Chem. 262, 17299–17303.
[11] Redpath, N.T., Foulstone, E.J. and Proud, C.G. (1996) EMBO J. 15, 2291–2297.
[12] Wang, L., Wang, X. and Proud, C.G. (2000) Am. J. Physiol. 278, 1056–1068.
[13] Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R. and Proud, C.G. (2001) EMBO J. 20, H4370–H4379.
[14] McLeod, L.E., Wang, L. and Proud, C.G. (2001) FEBS Lett. 489, 225–228.
[15] Redpath, N.T. (1992) Anal. Biochem. 202, 340–343.
[16] Diggle, T.A., Bloomberg, G.B. and Denton, R.M. (1995) Biochem. J. 306, 135–139.
[17] Flynn, A. and Proud, C.G. (1996) Eur. J. Biochem. 236, 40–47.
[18] Moule, S.K., Edgell, N.J., Welsh, G.I., Diggle, T.A., Foulstone, E.J., Heesom, K.J., Proud, C.G. and Denton, R.M. (1995) Biochem. J. 311, 595–601.
[19] Tee, A.R. and Proud, C.G. (2000) Oncogene 19, 3021–3031.
[20] Hawley, S.A., Gadalla, A.E., Olsen, G.S. and Hardie, D.G. (2002) Diabetes 51, 2420–2425.
[21] Bradford, M.M. (1976) Anal. Biochem. 77, 248–254.
[22] Hinnebusch, A.G. (2000) in: Translational Control of Gene Expression (Sonenberg, N., Hershey, J.W.B. and Mathews, M.B., Eds.), pp. 185–243, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[23] Atkinson, D.E. (1977) Cellular Energy Metabolism and its Regulation, Academic Press, New York.
[24] Casey, T.M., Dufall, K.G. and Arthur, P.G. (1999) Eur. J. Biochem. 261, 740–745.
[25] Marsin, A.-S., Bertrand, L., Rider, M.H., Deprez, J., Beauloye, C., Vincent, M.F., Van den Berghe, G., Carling, D. and Hue, L. (2000) Curr. Biol. 10, 1247–1255.
[26] Winder, W.W., Holmes, B.F., Rubink, D.S., Jensen, E.B., Chen, M. and Holloszy, J.O. (2002) J. Appl. Physiol. 88, 2219–2226.
[27] Horman, S., Browne, G.J., Krause, U., Patel, J.V., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C.G. and Rider, M.H. (2002) Curr. Biol. 12, 1419–1423.
[28] Munoz, F., Martin, M.E., Salinas, M. and Fando, J.L. (2001) FEBS Lett. 492, 156–159.
[29] Bolster, D.R., Crozier, S.J., Kimball, S.R. and Jefferson, L.S. (2002) J. Biol. Chem. 277, 23977–23980.
[30] Salt, I.P., Johnson, G., Ashcroft, S.J. and Hardie, D.G. (1998) Biochem. J. 335, 533–539.
[31] Leclerc, I., da Silva Xavier, G. and Rutter, G.A. (2002) Prog. Nucleic Res. Mol. Biol. 71, 69–90.