Biochimica et Biophysica Acta 1823 (2012) 800–807

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Evidence that AMP-activated protein kinase can negatively modulate Ornithine decarboxylase activity in cardiac myoblasts

Catherine L. Passariello [a,1], Davide Gottardi [a,1], Silvia Cetrullo [a], Maddalena Zini [a], Gabriele Campana [b], Benedetta Tantini [a], Carla Pignatti [a], Flavio Flamigni [a], Carlo Guarnieri [a], Claudio M. Caldarera [c], Claudio Stefanelli [a,*]

[a] Department of Biochemistry "G. Moruzzi", University of Bologna, Bologna, Italy
[b] Department of Pharmacology, University of Bologna, Bologna, Italy
[c] I.N.R.C. National Institute for Cardiovascular Research, Bologna, Italy

ARTICLE INFO

Article history:
Received 24 May 2011
Received in revised form 21 December 2011
Accepted 22 December 2011
Available online 31 December 2011

Keywords:
AMP-activated protein kinase (AMPK)
Ornithine decarboxylase (ODC)
Cardiac hypertrophy
Isoproterenol

ABSTRACT

The responses of AMP-activated protein kinase (AMPK) and Ornithine decarboxylase (ODC) to isoproterenol have been examined in H9c2 cardiomyoblasts, AMPK represents the link between cell growth and energy availability whereas ODC, the key enzyme in polyamine biosynthesis, is essential for all growth processes and it is thought to have a role in the development of cardiac hypertrophy. Isoproterenol rapidly induced ODC activity in H9c2 cardiomyoblasts by promoting the synthesis of the enzyme protein and this effect was counteracted by inhibitors of the PI3K/Akt pathway. The increase in enzyme activity became significant between 15 and 30 min after the treatment. At the same time, isoproterenol stimulated the phosphorylation of AMPKα catalytic subunits (Thr172), that was associated to an increase in acetyl coenzyme A carboxylase (Ser72) phosphorylation. Downregulation of both α1 and α2 isoforms of the AMPK catalytic subunit by siRNA to knockdown AMPK enzymatic activity, led to superinduction of ODC in isoproterenol-treated cardiomyoblasts. Downregulation of AMPKα increased ODC activity even in cells treated with other adrenergic agonists and in control cells. Analogue results were obtained in SH-SY5Y neuroblastoma cells transfected with a shRNA construct against AMPKα. In conclusion, isoproterenol quickly activates in H9c2 cardiomyoblasts two events that seem to contrast one another. The first one, an increase in ODC activity, is linked to cell growth, whereas the second, AMPK activation, is a homeostatic mechanism that negatively modulates the first. The modulation of ODC activity by AMPK represents a mechanism that may contribute to control cell growth processes.

1. Introduction

Cardiac hypertrophy and the subsequent progression towards heart failure represent a major cause of morbidity and mortality in industrialized countries. The defining features of cardiac hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and a higher degree of sarcomere organization [1]. These changes are preceded and accompanied by the re-induction of the so-called fetal cardiac gene program, characterized by a pattern of altered gene expression that mimics that observed during embryonic heart development [2]. Cardiac hypertrophy is also associated with a shift from fatty acids to glucose as the primary energy source, an additional feature in common with the fetal heart. A characteristic event in response to agents that induce cardiac hypertrophy is the upregulation of Ornithine decarboxylase (ODC), the key enzyme in the biosynthesis of polyamines. Polyamines have multiple roles in cardiac physiology [3] and a role for ODC and polyamine metabolism in cardiac hypertrophy was described since long ago [4]. Virtually all treatments that lead to cardiac hypertrophy cause an early induction of cardiac ODC either in the whole animal [4,5] and in cellular models [6–8]. In the classical model of adrenergic agonist-induced hypertrophy, pharmacological inhibition of ODC results in an attenuation of the hypertrophic response [4–6,9], suggesting a role of ODC in this process. More recently, targeted ODC overexpression in the heart was found to produce a moderate baseline cardiac hypertrophy in transgenic mice that also exhibited a greatly increased sensitivity to isoproterenol-induced hypertrophy [9]. On the other hand, ODC inhibition did not influence the expression of markers of hypertrophy in post-ischemic hearts [10] or following tiroxine treatment [11].

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a key metabolic regulator that is activated in response to

Abbreviations: ACC, Acetyl Coenzyme A Carboxylase; AMPK, AMP-Activated Protein Kinase; ANF, Atrial Natriuretic Factor; DFMO, α-difluoromethylornithine; ODC, Ornithine Decarboxylase

* Corresponding author. Fax: +39 0512091224.
E-mail address: claudio.stefanelli@unibo.it (C. Stefanelli).

[1] These authors contributed equally to this work.

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.12.013

metabolic stress coming from altered energy supply and/or demand. Additionally this enzyme can be activated by hormones that influence cellular metabolism, such as adiponectin and catecholamines [12]. The role of AMPK in cardiac hypertrophy is complex, and apparently contrasting findings are reported in literature. Many papers, reviewed in Refs. [13,14], have shown that AMPK has an anti-hypertrophic role. In this respect, strong evidence comes from the enhanced cardiac hypertrophy in genetic models with diminished heart AMPK activity [15,16] and from the fact that AMPK inhibits ATP consuming pathways such as protein synthesis [17]. On the other hand, activation of AMPK in hypertrophied hearts has been observed in a number of studies, reviewed in Refs. [14,18], and could be associated to the energetic requirements of hypertrophic heart [19–21]. Furthermore, adrenergic agonists cause an increase in AMPK activation in several cells and tissues, including different cardiac models [22–25], even if they are classical inducers of cardiac hypertrophy either *in vivo* or *in vitro*. In this light, the typical ODC induction triggered by adrenergic effector discussed above, seems to be in contrast with the well documented homeostatic role of AMPK that inhibits protein synthesis by interfering with the mTOR pathway [17,26]. To date, nothing is known about the possible interrelations between AMPK and ODC and we have now examined the response of the two enzymes to adrenergic stimulation in H9c2 heart cells, that represent a useful model in which molecular events associated to hypertrophy can be studied [27].

2. Materials and methods

2.1. Cell culture and treatment

H9c2 cardiomyoblasts (embryonic rat-heart derived cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Celbio) supplemented with 10% heat inactivated fetal calf serum (FCS), 5% glutamine and antibiotics as described [28]. Cardiomyocyte cultures were prepared from 1- to 3-day old newborn Wistar rat hearts, as described previously [8]. Before the treatments, the cells were serum starved for 18 h in medium containing 1% FCS. SH-SY5Y neuroblastoma cells were cultured in HAM'S F-12 / MEM containing 10% fetal bovine serum, 5% glutamine, 1% non-essential amino acids and antibiotics.

All treatments were dissolved in phosphate-buffered saline (PBS) or dimethylsulphoxide (DMSO) at 1000× concentration and added to cell cultures in order to obtain the required concentration in the medium. Control cells received the corresponding volume of the vehicle. Cell viability was determined by trypan blue exclusion by counting living cells and stained dead cells with a Burker hemocytometer.

2.2. Western blotting

H9c2 cells were collected in lysis buffer (5 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS, 0.1% Triton X-100, and protease inhibitors in 20 mM HEPES pH 7.5) and subjected to two cycles of freeze-thawing. The homogenate was then centrifuged at 15,000 ×*g* for 15 min and the supernatant, diluted in loading buffer (2% SDS, 5% glycerol, 0.002% bromophenol blue, 4% β-mercaptoethanol in 0.25 M Tris–HCl, pH 6.8), and then denatured by boiling for 4 min. Aliquots corresponding to 80 μg protein were analyzed by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and probed with the specific primary antibody. After further washing, the membrane was then incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Immunoreactive bands were visualized by chemiluminescence with the ECL reagent (Amersham). Antibodies against phospho-Acetyl Coenzyme A Carboxylase β (ACC) (Ser79 in human), AMPKα1/α2, phospho-AMPKα1/α2 (Thr172 in human), phospho-Akt (Ser473) were from Cell Signaling. Anti-Akt, anti-ACC, anti-GAPDH, and anti-β-actin were obtained from Santa Cruz. β-Actin or GAPDH was used as internal control. Quantitative assay of immunoblotting was obtained by densitometry with a Fluor-S Max MultiImager instrument (Bio-Rad).

2.3. ODC activity and expression

To measure the activity of ODC, the cells were washed with PBS and scraped in a buffer consisting of 0.1 mM EDTA, 0.02 mM pyridoxal phosphate, 1% Triton X-100, 5 mM dithiothreitol in 50 mM Tris-HCl buffer, pH 7.2. The cells were disrupted by freeze-thawing three times and then centrifuged at 11,000 rpm for 15 min. ODC activity in the supernatant was measured in duplicate by estimating the release of $^{14}CO_2$ from [^{14}C-Carboxyl]-ornithine during a 60 min incubation [28]. Specific ODC activity is expressed as units/mg protein, where 1 unit corresponds to 1 pmol of CO_2/min of incubation. The amount of protein in samples was determined by the Bradford method [29]. The level of ODC mRNA was measured by using real-time PCR. β-Actin was used as a housekeeping gene. Sample extraction and PCR details [25], and primers used in PCR [30] were previously described.

2.4. Cell metabolites

Acid-soluble metabolites were extracted in ice cold 0, 3 M perchloric acid and measured by HPLC. Separations were achieved by a 25 cm × 4.6 mm Gemini 5μ column from Phenomenex. Polyamines in cardiac cells were determined in acidic cellular extracts by reversed phase HPLC after derivatization with dansyl chloride [31]. The cellular content of ATP and other nucleotides was determined after extract neutralization with buffered 3 M KOH following conversion into fluorescent etheno-derivatives [32].

2.5. RNA interference

The sequences of short interfering RNAs (siRNA) directed against the catalytic subunits of rat AMPK (Obtained from Sigma-Genosys) were as follows: for AMPKα1, 5′-CUUAUUGGAUUUCCGAAGUTT-3′; for AMPKα2, 5′-GACAUUAUGGCGGAGGUGUTT-3′. In most experiments, a siRNA against Luciferase (Luc) was used as negative control (5′-AACUUACGCUGAGUACUUCTT-3′). In some cases, the control siRNA-A, purchased from Santa Cruz Biotechnology, was used. For transient transfection experiments, H9c2 cells at 50% of confluence were transfected with a final concentration of 100 nM siRNA for 24 h by Transfection reagent (Santa Cruz) according to manufacturer's instructions. Transfection in newborn rat cardiomyocytes was achieved as described [25]. Twenty-four hours after transfection, cells were then treated with the designated drugs. RNA interference by short hairpin RNA (shRNA) was obtained with constructs purchased from Sigma-Aldrich (MISSION® shRNA). SH-SY5Y cells were transfected with either a control shRNA or shRNA against the expression of AMPKα1 using the FuGene reagent (Roche) and subject to puromycin selection.

2.6. Hypertrophy experiments

The rate of protein synthesis in H9c2 cells was measured by [^{3}H] leucine incorporation in acid-insoluble fraction [33]. The protein content *per* cell was assayed as described by Hwang et al. [34]. The expression of the hypertrophy marker ANF was determined by real-time PCR as previously detailed [8], β-actin being used as housekeeping gene.

2.7. Data analysis

All the experiments shown were performed independently at least three times with comparable results. The blots are representative of

results obtained in multiple experiments. All the data presented in graphs are expressed as means ± S.E. of the mean of the indicated numbers of independent determinations. Statistical comparison between two groups was done by Student's unpaired two-tailed *t* test or one-way ANOVA analysis, when appropriate. Differences were considered as significant when $P<0.05$.

3. Results

Adrenergic agonists such as phenylephrine and isoproterenol cause a hypertrophic response in H9c2 cardiomyoblasts similar to that evoked in primary cardiomyocytes and in the whole heart [33–35]. The effect of isoproterenol on AMPK activation was firstly studied. Analysis of soluble extracts prepared from whole H9c2 cell lysates showed increased AMPKα (Thr172) phosphorylation following treatment with isoproterenol (10 μM). The experiment depicted in Fig. 1A shows that an increased phosphorylation of AMPKα catalytic subunits could be observed as soon as 15 min. This effect remained detectable for 2–3 h and was accompanied with the increased phosphorylation of its substrate ACC (Ser79). After 30 min of isoproterenol treatment, the AMPK phosphorylation level was increased by 1.9 ± 0.4 times with respect to control cells (n = 6, $P<0.05$), and that of ACC by 1.7 ± 0.2 times (n = 3, $P<0.05$). AMPK phosphorylation was also increased (Fig. 1B) by phenylephrine and by the physiological effector norepinephrine (both 10 μM), according to previous reports [23,36]. Pretreatment with the β-antagonist propranolol (10 μM) partially reduced AMPK phosphorylation in response to isoproterenol (Fig. 1C). A strong increase in AMPKα (Thr172) phosphorylation could be observed in cells incubated with oligomycin plus deoxyglucose and was associated with a sharp increase in the AMP/ATP ratio (Fig. 1D). The use of deoxyglucose was required because, in our hands, hypoxia or mitochondrial inhibitors alone scarcely affected adenylate nucleotide levels within 3 h, possibly as a consequence of the dependence of H9c2 cells on anaerobic glycolysis in these conditions, as evidenced by lactate accumulation in the medium. On the other hand, the effect of isoproterenol was not associated to any change in the level of ATP, ADP and AMP, and the AMP/ATP ratio was unchanged.

Treatment with isoproterenol increased ODC activity with a timing identical to that observed in rat cardiomyocytes [6] and in the whole heart [4]. The increase in enzyme activity was very rapid and became significant between 15 and 30 min after the treatment (Fig. 2A). After 4 h of treatment, putrescine, the product of ODC catalysis, was increased (Fig. 2B), whereas the major polyamines spermidine and spermine were unchanged (not shown). The increase in ODC activity was blocked by cycloheximide, an inhibitor of protein synthesis (Fig. 2C). ODC is a short-lived protein, whose level can be regulated by the rate of its degradation by the proteasome [37]. In the presence of the proteasome inhibitor MG132, basal ODC activity was increased as expected, however, isoproterenol continued to increase ODC activity (Fig. 2D), indicating that a change in protein stability was not the primary mechanism of ODC upregulation by isoproterenol. These data show that isoproterenol induced ODC activity by increasing the synthesis of new enzyme protein. Actually, the amount of ODC mRNA progressively increased after 1 h and 2 h following isoproterenol (Fig. 2E). ODC induction was blocked by propranolol (Fig. 2F).

ODC induction by isoproterenol was abolished in cells treated with the PI3K/Akt pathway inhibitors Wortmannin and LY294002 or with the dual PI3K/mTOR inhibitor BEZ235 (Fig. 3A). These inhibitors also significantly decreased the ODC basal activity in control cells, suggesting a central role of Akt-mediated signalling in the regulation of ODC. Akt (Ser473) phosphorylation was slightly but significantly increased following isoproterenol and after 30 min was increased 1.7 ± 0.2 (n = 4) times (Fig. 3B).

In isoproterenol-treated cells, inhibition of ODC activity by pretreatment with the irreversible and specific ODC inhibitor α-difluoromethylornithine (DFMO) (Fig. 4A) reduced the expression of the hypertrophy related gene ANF (Fig. 4B) and prevented the increase in [^{3}H]leucine incorporation into cellular proteins (Fig. 4C) and in the total protein content *per* cell (Fig. 4D). These data show that ODC activity is required because H9C2 cells exposed to isoproterenol exhibit the characteristics of hypertrophy.

To determine whether ODC activity could be influenced by AMPK, H9c2 cardiomyoblasts were exposed for 2 h to isoproterenol in the presence of AMPK inhibitors AraA [38], compound C [39] or STO-609 (Table 1). This last compound is a CAMKKβ inhibitor that in some cases can prevent AMPK activation, since CAMKKβ is an AMPK upstream kinase [12], but it has been proven to be also a powerful direct inhibitor of AMPK [40]. Compound C could be used only at

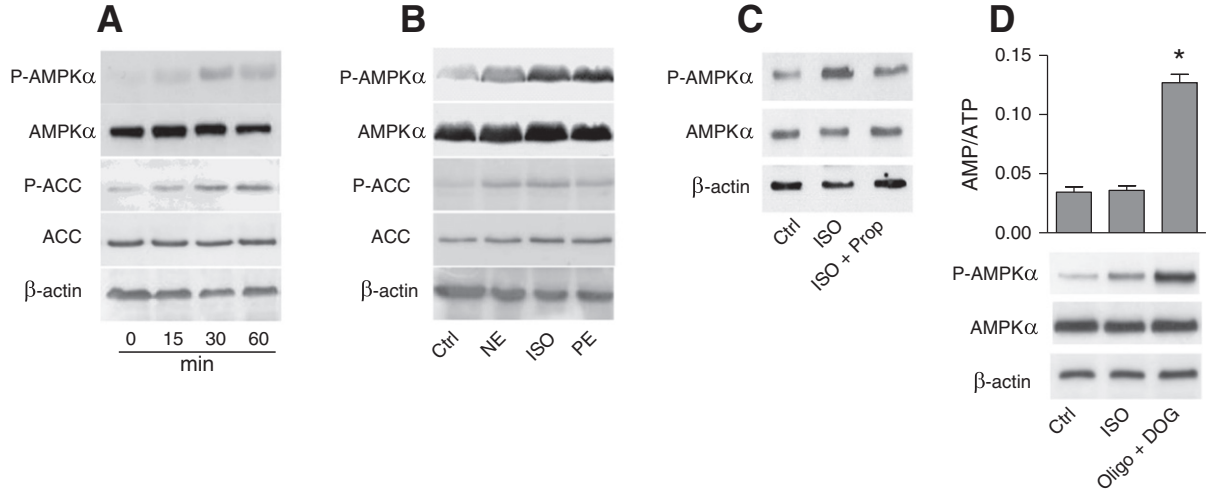

Fig. 1. Isoproterenol (ISO) increases AMPK phosphorylation in H9c2 cardiomyoblasts. (A) The cells were incubated in the presence of 10 μM isoproterenol for the indicated time. Cell lysates were analyzed by Western blotting with antibodies against α subunits of AMPK (AMPKα) or Thr172-phosphorylated α subunits of AMPK (P-AMPKα), or phospho-ACC (Ser79). (B) The cells were incubated for 1 h in the absence or presence of a 10 μM concentration of norepinephrine (NE), isoproterenol (ISO), phenylephrine (PE), then cell extracts were analyzed by Western blotting. (C) The phosphorylation of AMPKα was assayed in cells incubated 30 min with 10 μM isoproterenol with or without a pre-treatment for 30 min with 10 μM propranolol. (D) The cardiomyoblasts were incubated 60 min in the presence of 10 μM isoproterenol or oligomycin (20 μg/ml) plus 2 mM deoxyglucose (DOG), afterward cells were collected for the determination of adenylate nucleotide levels and AMPKα phosphorylation. In the graph reporting the AMP/ATP ratio, data are means ± S.E. of three measurements; *, $P<0.05$ vs. control cells.

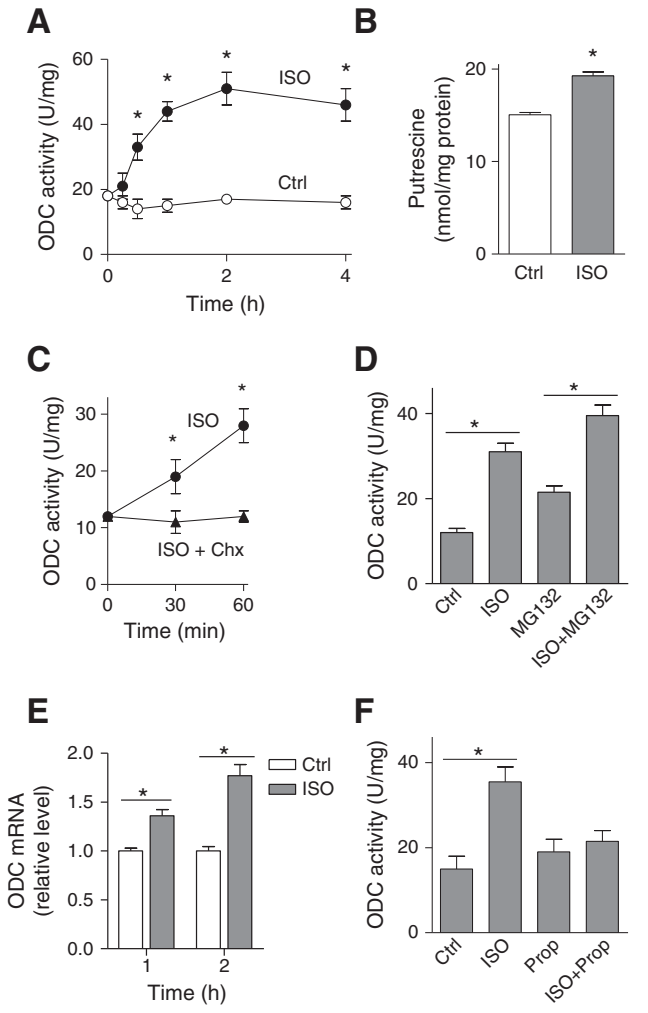

Fig. 2. Isoproterenol (10 μM, ISO) increases ODC activity and mRNA level in H9c2 cardiomyoblasts. (A) The cells were incubated in the absence or presence of isoproterenol for the indicated time. Cell lysates were then assayed for ODC activity. (B) The content of putrescine was measured by HPLC in acid extracts from cells incubated for 4 h in the absence or presence of isoproterenol. (C) H9c2 cells were treated with isoproterenol in the absence or presence of 0.2 mM cycloheximide (Chx) to inhibit protein synthesis. The cells were collected at the indicated time for the assay of ODC activity. (D) Control cells and isoproterenol-treated cells were incubated 2 h in the absence of presence of a 10 μM concentration of the proteasome inhibitor MG132. The cells were then collected and ODC was assayed in cell extracts. (E) ODC mRNA levels were measured at the indicated time after isoproterenol treatment by real-time PCR. (F) ODC activity was assayed in cells incubated 30 min with 10 μM isoproterenol with or without a pretreatment for 30 min with 10 μM propranolol. In all panels the presented results are means ± S.E. of three to five measurements. *, $P<0.05$ vs. control cells.

suboptimal concentration (1 μM) because at higher dosages it induced apoptosis. Interestingly, ODC activity resulted slightly increased in cells incubated in the presence of aforementioned AMPK inhibitors. On the other hand, the AMPK activator AICAR (4 mM) and especially metformin (5 mM) slightly decreased ODC activity. Phenformin, a more potent analogue of metformin, caused a dose dependent inhibition of ODC activity. These results suggest that AMPK activity could be associated to a decrease in ODC activity. On the contrary, ODC inhibition with 0.1 mM DFMO did not induce any effect on AMPK level or phosphorylation in either control cells and isoproterenol-treated cells (not shown).

Since the specificity of pharmacological inhibitors/activators always remains limited, to support the suggestion of a modulating effect of AMPK on ODC, a siRNA approach was used. The cells were transfected with two siRNAs respectively targeted against the α1 and α2 isoforms of the AMPK catalytic subunit in order to knockdown AMPK enzymatic activity, or with a siRNA directed against luciferase (control siRNA). The cardiac myoblasts were then exposed for 2 h to isoproterenol. Fig. 5A shows that a significant degree of reduction in AMPKα protein level of about 70% was obtained in cells pretreated with both siRNAs against α1 and α2 isoforms of AMPKα. This treatment also led to a significant reduction in cellular AMPK activity as shown by the decrease in the phosphorylation of ACC (Ser79), a physiological AMPK substrate. The knock-down of only one isoform of AMPKα did not influence ACC (Ser79) phosphorylation.

When both α1 and α2 isoforms of AMPK were silenced, along with the decrement in AMPK activity there was a marked increase in ODC activity in H9c2 cells treated with isoproterenol. siRNA-mediated downregulation of a single isoform had a very weak effect on ODC activity, but the effect of the siRNA against α2 isoform was somewhat more pronounced than that of the α1 siRNA. Fig. 5B shows that the pretreatment with α1 plus α2 siRNAs increased ODC activity also in response to adrenergic effectors norepinephrine or phenylephrine. AMPK downregulation increased ODC activity in control cells too. The treatment with α1 plus α2 siRNAs did not influence the expression of ERK 1/2 or Akt (not shown), that are associated to the growth promoting effect of adrenergic stimulation in cardiac cells [41,42].

Adrenergic agonists activate AMPK in several cardiac models and norepinephrine increases AMPKα (Thr172) phosphorylation in neonatal rat cardiomyocytes [25]. With the aim to confirm the modulating effect of AMPK on ODC in this somewhat more physiological model, cardiomyocytes were transfected with AMPK α1 plus α2 siRNAs before the exposure to the adrenergic effector (Fig. 6A and B). In primary cardiomyocytes the transfection efficiency was lower with respect to H9c2 cardiomyoblasts and AMPKα was decreased by 42 ± 7% (N = 4). Nevertheless, after AMPK downregulation also in rat cardiomyocytes it was possible to observe either the ODC superinduction in response to norepinephrine and the increased basal activity in control cells, even if this late difference did not reach the level of significance ($P = 0.078$).

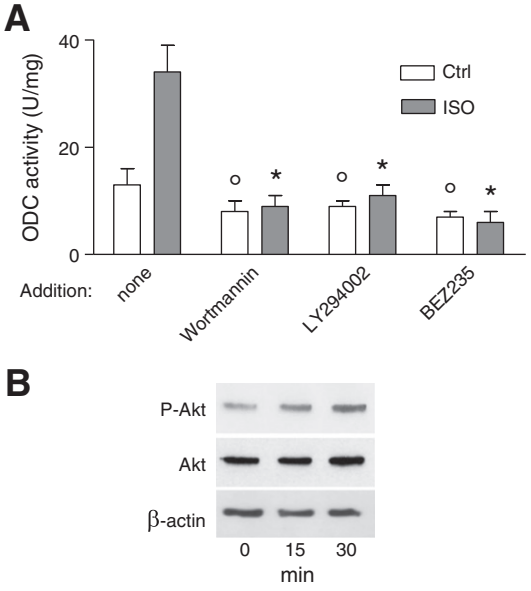

Fig. 3. Evidence that ODC activity in isoproterenol-treated cardiomyoblasts is regulated by signaling through Akt. (A) ODC activity was measured in extracts from H9c2 cardiomyoblasts incubated 2 h in the absence or presence of 10 μM isoproterenol (ISO) and the indicated compounds (2 μM wortmannin, 20 μM LY29004, 0.2 μM BEZ235). Results are means ± S.E. of three determinations. In isoproterenol treated cells: *, $P<0.05$ vs. cells incubated with isoproterenol only. In control cells: °, $P<0.05$ vs. cells incubated without any treatment. (B) The cells were incubated in the presence of 10 μM isoproterenol for the indicated time. Cell lysates were analyzed by Western blotting. The blots are representative of multiple experiments.

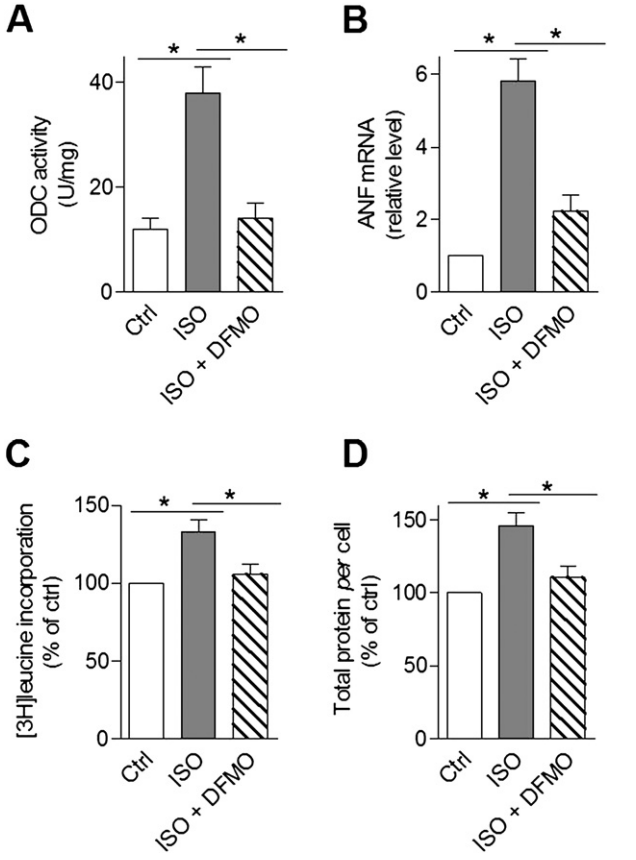

Fig. 4. Effect of ODC inhibition by DFMO on the development of hypertrophy in isoproterenol-treated H9c2 cardiomyoblasts. After seeding, H9c2 cells were pre-incubated in the absence or in the presence of 0.1 mM DFMO for 16 h. Then cells were treated with 10 μM isoproterenol (ISO). (A) ODC activity was determined after 2 h. (B) The expression of the hypertrophy marker ANF was assayed after 24 h by real time PCR and normalized against β-actin. (C) To determine the rate of protein synthesis, the cells were incubated for 24 h after isoproterenol in the presence of [^{3}H]leucine. (D) Total protein content *per* cell was measured after 48 h from isoproterenol. Results are means ± S.E. of three determinations. *, $P<0.05$ vs. cells incubated with isoproterenol only.

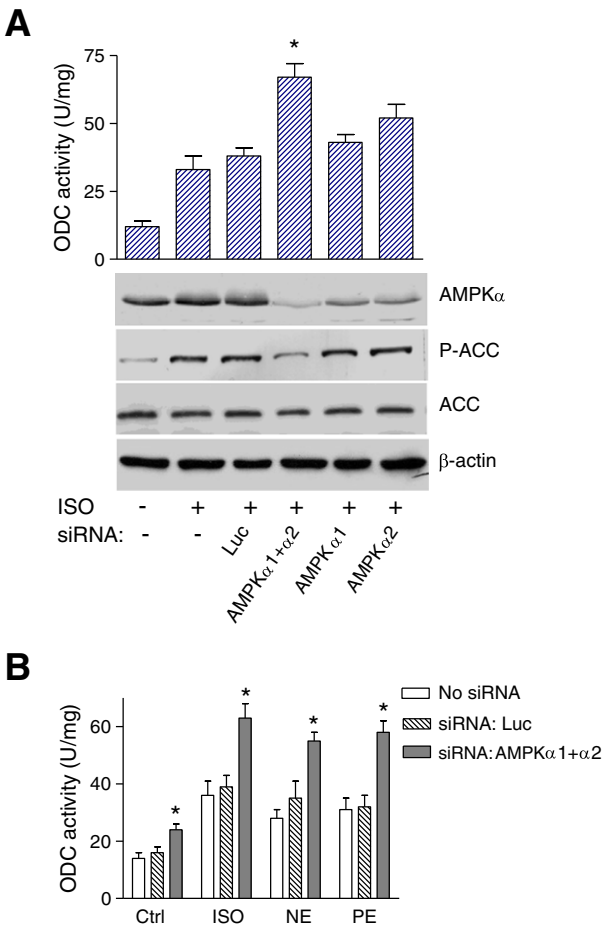

Fig. 5. AMPK knockdown causes an increase in ODC activity in isoproterenol-treated H9c2 cardiomyoblasts. (A) The cells were transfected with a control siRNA against luciferase (Luc), or AMPKα1 plus AMPKα2 siRNAs, or AMPKα1 siRNA, or AMPKα2 siRNA. The cells were then incubated in the absence or presence of 10 μM isoproterenol. The amount of AMPKα and phospho-ACC was determined after 1 h, whereas ODC activity was measured after 2 h of isoproterenol treatment. Results of ODC activity are means ± S.E. of three determinations, whereas the blots are representative of three experiments. *, $P<0.05$ vs. untransfected cells incubated with the same treatment. (B) H9c2 cells transfected with the luciferase siRNA (Luc) or AMPKα1 plus AMPKα2 siRNAs, were incubated for 2 h in the absence or presence of 10 μM norepinephrine (NE), or isoproterenol (ISO), or phenylephrine (PE), then ODC activity was measured in cell extracts. Results are means ± S.E. of three to five determinations. *, $P<0.05$ vs. untransfected cells incubated with the same treatment.

Lastly, in order to extend our finding and to give them further support, we wanted to assess whether the modulatory effect of AMPK on ODC activity was a characteristic of cellular models associated to hypertrophic growth or was a more general mechanism. In these experiments, AMPK downregulation by RNA interference was achieved with a different technique in a different cell line. SH-SY5Y human neuroblastoma cells were transfected with shRNA construct against AMPKα1, that represents by far the major AMPKα isoform in this cell line [43] and were then treated with isoproterenol. Again, the increase in ODC activity was higher in cells with a lower level of AMPK (Fig. 7). Also the basal ODC activity was significantly higher when AMPKα was knocked down. A similar response of basal ODC had been observed also in H9c2 cells and rat cardiomyocytes (see Figs. 5B and 6A) but in neuroblastoma cells appeared more pronounced.

Table 1
Effect of pharmacological AMPK inhibitors/activators on ODC activity in untreated and isoproterenol-treated H9c2 cardiomyoblasts.

Addition	Effect on AMPK	ODC activity (U/mg)	
		Ctrl	ISO
None (ctrl)	–	13 ± 3	34 ± 5
STO 609 (1 μM)	Inhibitor	16 ± 3	40 ± 4
AraA (0.5 mM)	Inhibitor	16 ± 2	41 ± 5
Compound C (1 μM)	Inhibitor	13 ± 2	37 ± 3
AICAR (4 mM)	Activator	11 ± 3	29 ± 4
Metphormin (5 mM)	Activator	9 ± 2*	23 ± 4*
Phenformin (0.5 mM)	Activator	12 ± 2	30 ± 4
Phenformin (1 mM)	Activator	9 ± 2*	26 ± 3*
Phenformin (2 mM)	Activator	8 ± 1*	21 ± 3*

ODC activity was determined in extracts from cells incubated 2 h in the absence or presence of 10 μM isoproterenol (ISO) and the indicated compounds. Results are means ± S.E. of three to six determinations. In isoproterenol treated cells: *, $P<0.05$ vs. cells incubated with isoproterenol only. In control cells: *, $P<0.05$ vs. cells incubated without any treatment.

4. Discussion

AMPK, which is allosterically activated by AMP and phosphorylated by upstream kinases, represents a link between cell growth and energy availability [12–14]. It is generally assumed that AMPK activates mechanisms that favour the formation of ATP both directly and indirectly, and blocks mechanism that consume ATP, such as protein synthesis [17]. Because of its homeostatic role, AMPK could essentially contrast the hyperplastic as well as hypertrophic growth [44]. The role of AMPK in cardiac hypertrophy however is not fully

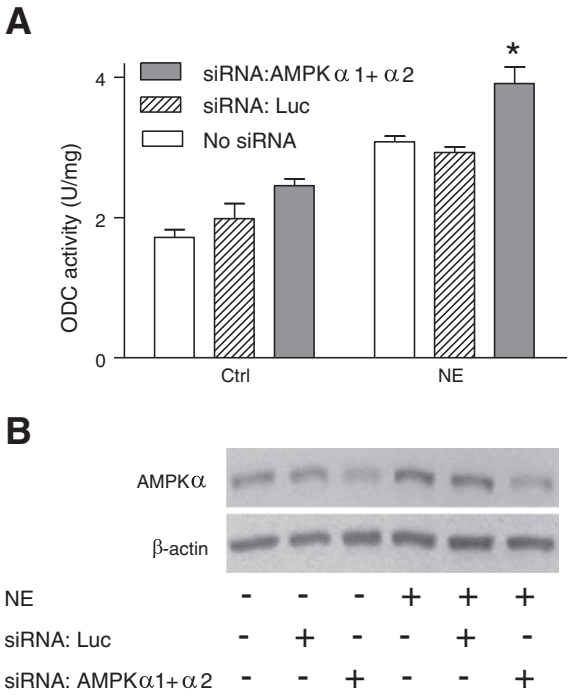

Fig. 6. AMPK knockdown causes an increase in ODC activity in norepinephrine-treated cardiomyocytes. (A) Neonatal rat cardiomyocytes were transfected with a control siRNA against luciferase (Luc), or with AMPKα1 plus AMPKα2 siRNAs. The cells were then incubated 2 h in the absence or presence of 10 μM norepinephrine (NE), afterward ODC activity was assayed. Results are means ± S.E. of three determinations. *, $P<0.05$ vs. untransfected cells incubated with the same treatment. (B) The amount of AMPKα was determined in the same experiment of panel A after 1 h of norepinephrine treatment. The blots are representative of three experiments.

understood. We have studied the influence of AMPK on growth-related events, by examining the responses of AMPK and ODC in a cell model of induced hypertrophy. Our findings have shown that treatment with adrenergic effectors such as isoproterenol quickly activates in H9c2 cardiomyoblasts two mechanisms that seem to contrast one another. The first one, increase in ODC activity, is linked to cell growth, whereas the second, AMPK activation, is a homeostatic mechanism that negatively modulates the first.

Isoproterenol reportedly causes AMPK activation in 3 T3-L1 preadipocytes [45], in brown adipocytes [46], in the *pectoralis* muscle of chick [47], in rat heart [22], in neonatal rabbit heart [24]. In H9c2 cells, Xu et al. [23] did not see a significant effect of isoproterenol within 10 min, but we have observed that the increase in AMPKα (Thr172) phosphorylation becomes well evident after 15–30 min of treatment and is associated to increased enzyme activity, as shown by the elevation in ACC phosphorylation. Boone et al. [48] have shown that at very early times (2–3 min) after isoproterenol, protein kinase A (PKA) has a role more pronounced than AMPK in the increase in ACC phosphorylation. However, the downregulation of AMPK by siRNA causes a significant decrease in ACC (Ser79) phosphorylation after 30–60 min of exposure to isoproterenol, suggesting that at these times AMPK is the main ACC kinase. Isoproterenol does not increase the AMP/ATP ratio, but it is known that AMPK can be activated by several alternative pathways; for example, AMPK (Thr172) phosphorylation can increase in response to calcium or reactive oxygen species [12]. Adrenergic stimulation activates AMPK in many cell types with mechanisms that may be very complex [49] independently of the effect on cell growth, but possibly to prepare the cells for increased energetic requirements [19,20]. According with this view, and only apparently in contrast with other reports, recently it has been published that adrenergic signaling can inactivate AMPK in adult not-beating quiescent rat cardiomyocytes [50] in which, as pointed by the authors, the signaling pathways are very different than those in contracting cardiomyocytes.

ODC is the enzyme responsible for the biosynthesis of putrescine, the precursor of aliphatic polyamines [37]. Polyamines are critical in the cell cycle, showing a marked increase during all cellular growth processes [51]. Isoproterenol induces ODC activity in H9c2 cells by increasing the level of ODC mRNA and ultimately promoting the synthesis of the enzyme protein. ODC is one of the most finely regulated enzymes [37] and its cellular activity results from the integration of many signals. ODC synthesis can be modulated by the PI3K/Akt pathway and downstream by the mTOR/P70S6K axis [52–54], and these pathways are known to be activated by isoproterenol in cardiomyocytes [55–57]. In H9c2 cells, Jeong et al. did not observe change in Akt phosphorylation 48 h after isoproterenol, when morphological aspects of hypertrophy were established [35], but Yano et al. reported a rapid activation within minutes [57]. In our experiments, isoproterenol causes a rapid increase in Akt phosphorylation, according to previous reports [56,57], that is associated to ODC induction. Pharmacological inhibition of the PI3K/Akt pathway blocks ODC induction by isoproterenol and also reduces the basal ODC activity in control cells (Fig. 3), underlining the importance of Akt-mediated signaling in ODC regulation. Inhibition of mTOR is an important aspect of AMPK function [26] and it is thought to represent the main mechanism of the anti-hypertrophic effect of AMPK [12,17]. So it seems reasonable to think that this is probably also the mechanism responsible of the negative modulation of ODC. AMPK downregulation increases ODC induction by isoproterenol even in rat cardiomyocytes. Importantly, also basal ODC activity results higher when AMPK activity is decreased, either in heart-derived cells as well as in unrelated neuroblastoma cells, suggesting a wide involvement of AMPK in ODC modulation. Actually, the basal degree of AMPKα (Thr172) and ACC (Ser79) phosphorylation in control cells indicates a constitutive basal level of AMPK activity that can be involved in the regulation of cellular processes.

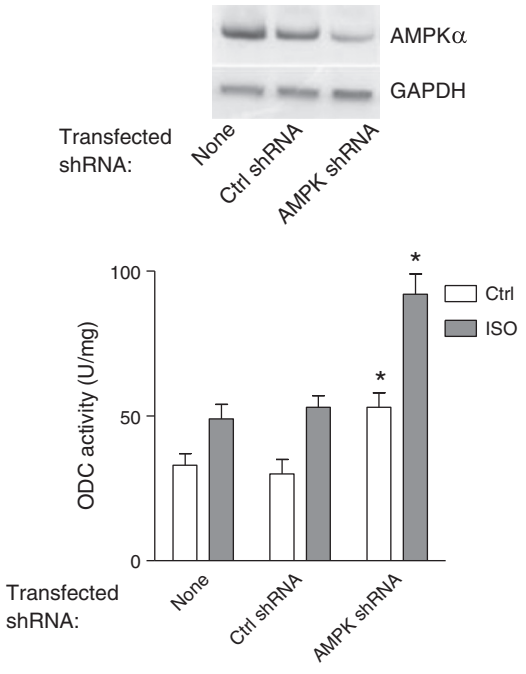

Fig. 7. AMPK downregulation by shRNA increases ODC activity in SH-SY5Y neuroblasoma cells. The cells were transfected with constructs producing control shRNA or a shRNA inhibiting AMPKα1 expression (AMPK shRNA). The blot shows the level of AMPK in untransfected and transfected cells. The lower panel shows the effect of treatment with 10 μM isoproterenol (ISO) for 2 h on ODC activity. Results are means ± S.E. of three determinations. *, $P<0.05$ vs. untransfected cells incubated with the same treatment.

Polyamines are generally thought to have a role in cardiac hypertrophy [3–9], and we have shown that ODC inhibition counteracts the development of hypertrophic phenotype in isoproterenol-treated H9c2 cardiomyoblasts. An obvious question is whether the modulatory effect of AMPK on ODC represents a mechanism that can contribute to control the developing of hypertrophy. Interestingly, in AMPKα2 knockout mice, p70S6K is constitutively overstimulated and the mice develop an exaggerated myocardial hypertrophic response to isoproterenol [16], similar to that observed in mice with targeted overexpression of ODC in the heart [9]. However, as a cautionary note, it should be noted that the precise role of ODC in cardiac hypertrophy is not completely defined; hence further work is required to answer the question. In this respect it would be of interest to determine ODC activity and expression in the heart of AMPK knockout mice.

Another question is why two apparently contrasting mechanisms are activated at the same time in heart cells committed to hypertrophy. We think that the rapid activation of AMPK following adrenergic stimuli is probably due to the cells effort to guarantee itself energetic substrates in order to respond to an increase in ATP demand. The lack of optimal nutritional conditions (energetic stress) leading to a prolonged activation of AMPK could instead contrast the establishment of hypertrophy, possibly also by means of the negative modulation of ODC. We have observed that AMPK downregulation affects ODC activity even in neuroblastoma cells that obviously are not associated to hypertrophic growth. Therefore, more in general, the ODC regulatory action of AMPK may be one of the mechanisms by which cells couple the control of cell growth with nutrients availability. In conclusion, we suggest that the modulation of ODC activity by AMPK represents a self-regulatory mechanism that can participate to the regulation of cell growth processes.

Acknowledgements

This work was supported by the Italian MIUR (PRIN funding) and INRC (National Institute for Cardiovascular Research).

References

[1] A.H. Gradman, F. Alfayoumi, From left ventricular hypertrophy to congestive heart failure: management of hypertensive heart disease, Prog. Cardiovasc. Dis. 48 (2006) 326–341.
[2] H. Taegtmeyer, S. Sen, D. Vela, Return to the fetal gene program: a suggested metabolic link to gene expression in the heart, Ann. N. Y. Acad. Sci. 1188 (2010) 191–198.
[3] E. Giordano, F. Flamigni, C. Guarnieri, C. Muscari, C. Pignatti, C. Stefanelli, B. Tantini, C.M. Caldarera, Polyamines in cardiac physiology and disease, Open Heart Fail. J. 3 (2010) 25–30.
[4] J. Bartolome, J. Huguenard, T.A. Slotkin, Role of ornithine decarboxylase in cardiac growth and hypertrophy, Science (Washington, D.C.) 210 (1980) 793–794.
[5] J.C. Cubria, R. Reguera, R. Balana-Fouce, C. Ordonez, D. Ordonez, Polyamine-mediated heart hypertrophy induced by clenbuterol in the mouse, J. Pharm. Pharmacol. 50 (1998) 91–96.
[6] K.D. Schlüter, K. Frischkopf, M. Flesch, S. Rosenkranz, G. Taimor, H.M. Piper, Central role for ornithine decarboxylase in beta-adrenoceptor mediated hypertrophy, Cardiovasc. Res. 45 (2000) 410–417.
[7] M. Schäfer, K. Frischkopf, G. Taimor, H.M. Piper, K.D. Schlüter, Hypertrophic effect of selective beta(1)-adrenoceptor stimulation on ventricular cardiomiopatia from adult rat, Am. J. Physiol. Cell Physiol. 279 (2000) C495–C503.
[8] S. Cetrullo, A. Facchini, I. Stanic, B. Tantini, C. Pignatti, C.M. Caldarera, F. Flamigni, Difluoromethylornithine inhibits hypertrophic, pro-fibrotic and pro-apoptotic actions of aldosterone in cardiac cells, Amino Acids 38 (2010) 525–531.
[9] L.M. Shantz, D.J. Feith, A.E. Pegg, Targeted overexpression of ornithine decarboxylase enhances beta-adrenergic agonist-induced cardiac hypertrophy, Biochem. J. 358 (2001) 25–32.
[10] C. Mörlein, R. Schreckenberg, K.D. Schlüter, Ornithine decarboxylase activity modifies apoptotic and hypertrophic marker expression in post-ischemic hearts, Open Heart Fail. J. 3 (2010) 31–36.
[11] A.E. Pegg, Effect of α-difluoromethylornithine on cardiac polyamine content and hypertrophy, J. Mol. Cell. Cardiol. 13 (1981) 881–887.
[12] G.R. Steinberg, B.E. Kemp, AMPK in health and disease, Physiol. Rev. 89 (2009) 1025–1078.
[13] A.K.F. Wong, J. Howie, J.R. Petrie, C.C. Lang, AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease, Clin. Sci. (Lond) 116 (2009) 607–620.
[14] A.S. Kim, E.J. Miller, L.H. Young, AMP-activated protein kinase: a core signalling pathway in the heart, Acta Physiol. 196 (2009) 37–53.
[15] R. Shibata, N. Ouchi, M. Ito, S. Kihara, I. Shiojima, D.R. Pimentel, M. Kumada, K. Sato, S. Schiekofer, K. Ohashi, T. Funahashi, W.S. Colucci, K. Walsh, Adiponectin-mediated modulation of hypertrophic signals in the heart, Nat. Med. 10 (2004) 1384–1389.
[16] E. Zarrinpashneh, C. Beauloye, A. Ginion, A.C. Pouleur, X. Havaux, L. Hue, B. Viollet, J.L. Vanoverschelde, L. Bertrand, AMPKα2 counteracts the development of cardiac hypertrophy induced by isoproterenol, Biochem. Biophys. Res. Commun. 376 (2008) 677–681.
[17] A.Y. Chan, J.R. Dyck, Activation of AMP-activated protein kinase (AMPK) inhibits protein synthesis: a potential strategy to prevent the development of cardiac hypertrophy, Can. J. Physiol. Pharmacol. 83 (2005) 24–28.
[18] M. Arad, C.E. Seidman, J.G. Seidman, AMP-activated protein kinase in the heart. Role during health and disease, Circ. Res. 100 (2007) 474–488.
[19] M.F. Allard, H.L. Parsons, R. Saeedi, R.B. Wambolt, R. Brownsey, AMPK and metabolic adaptation by the heart to pressure overload, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) H140–H148.
[20] L. Nascimben, J.S. Ingwall, B.H. Lorell, I. Pinz, V. Schultz, K. Tornheim, R. Tian, Mechanisms for increased glycolysis in the hypertrophied rat heart, Hypertension 44 (2004) 662–667.
[21] R. Saeedi, V.V. Saran, S.S.Y. Wu, E.S. Kume, K. Paulson, A.P.K. Chan, H.L. Parsons, R.B. Wambolt, J.R.B. Dyck, R.W. Brownsey, M.F. Allard, AMP-activated protein kinase influences metabolic remodeling in H9c2 cells hypertrophied by arginine vasopressin, Am. J. Physiol. Heart Circ. Physiol. 296 (2009) H1822–H1832.
[22] D. An, G. Kewalramani, D. Qi, T. Pulinilkunnil, S. Ghosh, A. Abrahani, R. Wambolt, M. Allard, S.M. Innis, B. Rodrigues, β-Agonist stimulation produces changes in cardiac AMPK and coronary lumen LPL only during increased workload, Am. J. Physiol. Endocrinol. Metab. 288 (2005) E1120–E1127.
[23] M. Xu, Y.T. Zhao, Y. Song, T.P. Hao, Z.Z. Lu, Q.D. Han, S.Q. Wang, Y.Y. Zhang, α1-adrenergic receptors activate AMP-activated protein kinase in rat hearts, Acta Physiol. Sin. 59 (2007) 175–182.
[24] J.S. Jaswal, C.R. Lund, W. Keung, D.L. Beker, I.M. Rebeyka, G.D. Lopaschuk, Isoproterenol stimulates 5′-AMP-activated protein kinase and fatty acid oxidation in neonatal hearts, Am. J. Physiol. Heart Circ. Physiol. 299 (2010) H1135–H1145.
[25] S. Cetrullo, B. Tantini, A. Facchini, C. Pignatti, C. Stefanelli, C.M. Caldarera, F. Flamigni, A pro-survival effect of polyamine depletion on norepinephrine-mediated apoptosis in cardiac cells: role of signaling enzymes, Amino Acids 40 (2011) 1127–1137.
[26] R.J. Shaw, LKB1 and AMP-activated protein kinase control of mTOR signalling and growth, Acta Physiol. (Oxf) 196 (2009) 65–80.
[27] S.J. Watkins, G.M. Borthwick, H.M. Arthur, The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro, In Vitro Cell. Dev. Biol. Anim. 47 (2011) 125–131.
[28] B. Tantini, E. Fiumana, S. Cetrullo, C. Pignatti, F. Bonavita, L.M. Shantz, E. Giordano, C. Muscari, F. Flamigni, C. Guarnieri, C. Stefanelli, C.M. Caldarera, Involvement of polyamines in apoptosis of cardiac myoblasts in a model of simulated ischemia, J. Mol. Cell. Cardiol. 40 (2006) 775–782.
[29] J.E. Noble, M.J.A. Bailey, Quantitation of protein, Methods Enzymol. 463 (2009) 73–95.
[30] F. Flamigni, A. Facchini, C. Capanni, C. Stefanelli, B. Tantini, C.M. Caldarera, p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells, Biochem. J. 341 (1999) 363–369.
[31] C. Ventura, C. Ferroni, F. Flamigni, C. Stefanelli, M.C. Capogrossi, Polyamine effects on $[Ca^{2+}]_i$ homeostasis and contractility in isolated rat ventricular cardiomyocytes, Am. J. Physiol. Heart Circ. Physiol. 267 (1994) H587–H592.
[32] E.R. Lazarowski, R. Tarran, B.R. Grubb, C.A. van Heusden, S. Okada, R.C. Boucher, Nucleotide release provides a mechanism for airway surface liquid homeostasis, J. Biol. Chem. 279 (2004) 36855–36864.
[33] C. Villeneuve, A. Caudrillier, C. Ordener, N. Pizzinat, A. Parini, J. Mialet-Perez, Dose-dependent activation of distinct hypertrophic pathways by serotonin in cardiac cells, Am. J. Physiol. Heart Circ. Physiol. 297 (2009) H821–H828.
[34] G.S. Hwang, K.S. Oh, H.N. Koo, H.W. Seo, K.H. You, B.H. Lee, Effects of KR-31378, a novel ATP-sensitive potassium channel activator, on hypertrophy of H9c2 cells and on cardiac dysfunction in rats with congestive heart failure, Eur. J. Pharmacol. 540 (2006) 131–138.
[35] K. Jeong, H. Kwon, C. Min, Y. Pak, Modulation of the caveolin-3 localization to caveolae and STAT3 to mitochondria by catecholamine-induced cardiac hypertrophy in H9c2 cardiomyoblasts, Exp. Mol. Med. 41 (2009) 226–235.
[36] C.L. Passariello, M. Zini, P.A. Nassi, C. Pignatti, C. Stefanelli, Upregulation of SIRT1 deacetylase in phenylephrine-treated cardiomyoblasts, Biochem. Biophys. Res. Commun. 407 (2011) 512–516.
[37] A.E. Pegg, Regulation of ornithine decarboxylase, J. Biol. Chem. 281 (2006) 14529–14532.
[38] N. Henin, M.F. Vincent, G. Van den Berghe, Stimulation of rat liver AMP-activated protein kinase by AMP analogues, Biochim. Biophys. Acta 197 (1996) 197–203.
[39] L. Al-Khalili, A.V. Chibalin, M. Yu, B. Sjödin, C. Nylén, J.R. Zierath, A. Krook, MEF2 activation in differentiated primary human skeletal muscle cultures requires coordinated involvement of parallel pathways, Am. J. Physiol. Cell Physiol. 286 (2004) C1410–C1416.
[40] J. Bain, L. Plater, M. Elliott, N. Shapiro, C.J. Hastie, H. McLauchlan, I. Klevernic, J.S. Arthur, D.R. Alessi, P. Cohen, The selectivity of protein kinase inhibitors: a further update, Biochem. J. 408 (2007) 297–315.
[41] B.A. Rose, T. Force, Y. Wang, Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale, Physiol. Rev. 90 (2010) 1507–1546.

[42] A. Clerk, P.H. Sugden, Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists, Am. J. Cardiol. 83 (1999) 64H–69H.
[43] C. Thornton, A. Sardini, D. Carling, Muscarinic receptor activation of AMP-activated protein kinase inhibits orexigenic neuropeptide mRNA expression, J. Biol. Chem. 283 (2008) 17116–17122.
[44] S. Fogarty, D.G. Hardie, Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer, Biochim. Biophys. Acta 1804 (2010) 581–591.
[45] W. Yin, J. Mu, M.J. Birnbaum, Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis In 3T3-L1 adipocytes, J. Biol. Chem. 278 (2003) 43074–43080.
[46] D.S. Hutchinson, E. Chernogubova, O.S. Dallner, B. Cannon, T. Bengtsson, Beta-adrenoceptors, but not alpha-adrenoceptors, stimulate AMP-activated protein kinase in brown adipocytes independently of uncoupling protein-1, Diabetologia 48 (2005) 2386–2395.
[47] R. Joubert, S. Métayer Coustard, Q. Swennen, V. Sibut, S. Crochet, E. Cailleau-Audouin, J. Buyse, E. Decuypere, C. Wrutniak-Cabello, G. Cabello, S. Tesseraud, A. Collin, The beta-adrenergic system is involved in the regulation of the expression of avian uncoupling protein in the chicken, Domest. Anim. Endocrinol. 38 (2010) 115–125.
[48] A.N. Boone, B. Rodrigues, R.W. Brownsey, Multiple-site phosphorylation of the 280 kDa isoform of acetyl-CoA carboxylase in rat cardiac myocytes: evidence that cAMP-dependent protein kinase mediates effects of beta-adrenergic stimulation, Biochem. J. 341 (1999) 347–354.
[49] T. Pulinilkunnil, H. He, D. Kong, K. Asakura, O.D. Peroni, A. Lee, B.B. Kahn, Adrenergic regulation of AMP-activated protein kinase in brown adipose tissue in vivo, J. Biol. Chem. 286 (2011) 8798–8809.
[50] Y. Tsuchiya, F.C. Denison, R. Heath, D. Carling, D. Saggerson, 5'-AMP-activated protein kinase is inactivated by adrenergic signalling in adult cardiac myocytes, Biosci. Rep. 32 (2012) 197–209.
[51] E.W. Gerner, F.L. Meyskens Jr., Polyamines and cancer: old molecules, new understanding, Nat. Rev. Cancer 4 (2004) 781–792.
[52] F. Flamigni, S. Marmiroli, C. Capanni, C. Stefanelli, C. Guarnieri, C.M. Caldarera, Phosphatidylinositol 3-kinase is required for the induction of ornithine decarboxylase in leukemia cells stimulated to growth, Biochem. Biophys. Res. Commun. 239 (1997) 729–733.
[53] S.R. Kimball, L.M. Shantz, R.L. Horetsky, L.S. Jefferson, Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6, J. Biol. Chem. 274 (1999) 11647–11652.
[54] L.M. Shantz, Transcriptional and translational control of ornithine decarboxylase during Ras transformation, Biochem. J. 377 (2004) 257–264.
[55] A. Simm, K. Schlüter, C. Diez, H.M. Piper, J. Hoppe, Activation of p70(S6) kinase by beta-adrenoceptor agonists on adult cardiomyocytes, J. Mol. Cell. Cardiol. 30 (1998) 2059–2067.
[56] C. Morisco, D. Zebrowski, G. Condorelli, P. Tsichlis, S.F. Vatner, J. Sadoshima, The Akt-glycogen synthase kinase 3β pathway regulates transcription of atrial natriuretic factor induced by β-adrenergic receptor stimulation in cardiac myocytes, J. Biol. Chem. 275 (2000) 14466–14475.
[57] N. Yano, V. Ianus, T.C. Zhao, A. Tseng, J.F. Padbury, Y.T. Tseng, A novel signaling pathway for beta-adrenergic receptor-mediated activation of phosphoinositide 3-kinase in H9c2 cardiomyocytes, Am. J. Physiol. Heart Circ. Physiol. 293 (2007) H385–H393.