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Original Article

The activation of AMPK in cardiomyocytes at the very early stage of hypoxia relies on an adenine nucleotide-independent mechanism

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Abstract: The energy status of a cell plays a key role in its survival, and the exposure of eukaryotic cells to the hypoxia that accompanies the depletion of intracellular ATP triggers specific systemic adaptive responses. AMP-activated protein kinase (AMPK) has emerged as a key regulator of energy metabolism in the heart and plays a critical role in inducing these responses. However, the specific mechanism responsible for AMPK activation in cardiomyocytes at very early stages of hypoxia remain unclear. The goals of this study were to assess the relative contribution to AMPK activation of phosphorylation by AMPK kinase (AMPKK) and of positive allosterism due to AMP:ATP ratios in the early stages of hypoxia. Our results demonstrated that, compared with normoxic controls, neither intracellular AMP concentrations nor AMP:ATP ratios significantly increased within 1h of hypoxia onset. In contrast, a SAMS peptide phosphorylation assay and an immunoblot analysis revealed significant increases in both AMPK activity and ACC phosphorylation within 5min of hypoxic treatment. Furthermore, exposure of cardiomyocytes to hypoxia significantly increased AMPK phosphorylation within 5min, by 3- to 4-fold compared with controls ($P<0.01$), while overall levels of AMPKα protein did not differ between aerobic and anoxic cardiomyocytes. We also observed increased AMPKK activity in anoxic cardiomyocytes, through use of an α_{312} substrate. Taken together, our findings demonstrate that in the early stage of hypoxia in cardiomyocytes, increases in AMPK activity occur prior to and independently of increases in AMP concentration or in the AMP:ATP ratio. Instead, under these circumstances, AMPK is primarily activated by phosphorylation of the conserved Thr-172 residue in its activation loop by its upstream kinase AMPKK.

Keywords: Cardiomyocytes, hypoxia, AMPK, AMP, AMPK kinase

Introduction

The energy status of a cell plays a key role in its survival, and the exposure of eukaryotic cells to the hypoxia that accompanies the depletion of intracellular ATP triggers specific systemic adaptive responses. AMP-activated protein kinase (AMPK), a heterotrimeric enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ), has a critical function as a cellular energy sensor [1-3]. AMPK is allosterically activated by increases in the ratio of AMP to ATP [4, 5], which occur under conditions of cellular stress or energy deficiency, such as hypoxia, ischemia, and glucose deprivation [6, 7]. AMPK is also activated via phosphorylation of a conserved threonine in its activation loop (Thr172) by upstream kinases. Although positive allosterism is central to the regulation of AMPK, it has also been found that changes in AMPK activity following ischemia, insulin, metformin or hyperosmotic stress, do not changes in the AMP:ATP ratio [8-10]. In addition, AMPK has an important role in cardiovascular protection.

The pathway responsible for activating AMPK in cardiomyocytes under hypoxic conditions remains uncertain. The goals of the present study were to assess the relative contribution of phosphorylation by AMPK kinase (AMPKK) and of positive allosterism due to AMP:ATP ratios in stimulating AMPK during the very early stage of hypoxia.

Materials and methods

Reagents

Primary antibodies used in this study were rabbit anti-phospho-α-AMPK (Thr-172), and rabbit anti-α-AMPK, from New England Biolabs. Goat anti-actin (I-19) primary antibody, and goat anti-rabbit and donkey anti-goat secondary antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-phospho-acetyl-CoA carboxylase (Ser-79) antibody was purchased from Upstate Biotechnology. Secondary antibodies coupled to horseradish peroxidase were from Pierce. Dulbecco's modified Eagle's medium (DMEM) and HAM F12 media, fetal bovine serum, and mammalian protease inhibitor and phosphatase inhibitor mixtures were all purchased from Sigma.

Cell cultures

Neonatal rat ventricular myocytes (NRVMs) were prepared according to the method described by Wright *et al*. [11] with minor modifications. Ventricles of 1–3 day-old Wistar rats were minced and digested in phosphate-buffered saline (PBS) containing 0.1% trypsin (Gibco BRL) and 0.05% type I collagenase (Gibco BRL) at 37°C. The cells were pelleted by low speed centrifugation and suspended in DMEM containing 15% fetal bovine serum. A single preplating step was used to further increase the ratio of cardiomyocytes to noncardiomyocytes; noncardiomyocytes attached readily to the bottom of the culture dishes. The remaining unattached viable cells were collected and seeded into 25 cm^2 flask (2×10^6 cell/dish) and incubated. The cells were then incubated with DMEM supplemented with 20% fetal calf serum plus 0.1mmol/L 5-bromo-2-deoxyuridine for 72 hours to prevent low-level nonmyocardial cell proliferation, and then replaced with DMEM plus 20% calf serum. Experiments were performed 3–4 days after cell plating. The medium was changed 1h before hypoxic or normoxic treatment to ensure an adequate amount of nutrients and growth factors. All procedures were approved by the Third Military Medical University Animal Care and Use Committee.

Cell treatments

The hypoxia groups were incubated in a humidified Napco incubator maintained at 1% O_2 and 5% CO_2 with a balance of nitrogen for the indicated time periods. The normoxia group was incubated under standard cell culture conditions (95% air/5% CO_2). All cells were grown at 37°C.

High energy phosphate measurements

AMP and ATP contents were determined in neutralized perchloric acid extracts of cardiomyocytes with or without hypoxia treatment by high performance liquid chromatography, as described previously [12, 13].

Protein extraction and western blotting

Cells were rinsed twice with ice-cold PBS and scraped with lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin), Cells were lysed for 15 min on ice and then centrifuged at 14,000 x *g* for 10 min at 4°C. The protein concentration of the supernatant was determined, after which samples were subjected to SDS-PAGE and immunoblot analysis. Equal amounts of cleared homogenate were boiled before being subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% milk/1x Tris-buffered saline/0.1% Tween 20 and probed first with anti-AMPKα-pan antibody, anti-phosphoThr-172 AMPK antibody, anti-phosphoSer-79 ACC antibody, or anti-ACC antibody (all from Cell Signaling Technologies), and then probed with a horseradish peroxidase-coupled goat anti-rabbit secondary antibody (Jackson ImmunoResearch). Blots were developed using enhanced chemiluminescence reagent (Amersham Biosciences). Detected immunocomplexes were scanned using a calibrated densitometer (Bio-Rad) and quantified using Quantity One software (Bio-Rad).

AMPK activity assay

AMPK activity was measured using the SAMS peptide HMRSAMSGLHLVKRR phosphorylation assay. Cardiomyocyte lysates were readjusted to contain 0.8 mM DTT and 0.2 mM AMP, with or without 0.2 mM SAMS peptide [14, 15]. The kinase assay was performed in the presence of 5 mM $MgCl_2$, 0.2 mM ATP, and [^{32}P]ATP (New England Nuclear, Boston, MA) for 10 min at 37°C. Aliquots of the reaction mixture superna-

tant were spotted on Whatman filter paper (P81). The filters were washed with cold 150 mM phosphoric acid for 40 min and with acetone for 20 min. and allowed to dry before scintillation counting. AMPK activity was calculated as picomoles per milligram PEG-precipitated protein per minute, and results were expressed as the degree of increase compared with control.

Recombinant protein expression and purification

The C-terminal truncation mutant of the AMPK α1 subunit encoding amino acids 1–312 (α_{312}) was amplified from the full-length rat α1 cDNA (a gift from Dr. D Carling, Imperial College of London) by polymerase chain reaction. The sense and antisense primers used were: 5'-GTCGTACCATGGCCGAGAAGCAGAGCACGA-3' and 5'-CACTAGCTCGAGGTACAGGCAGCTGAGGA-3', respectively. The amplicon was cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into the bacterial expression vector pET30a using the restriction sites NcoI and XhoI. The resulting construct (α_{312}-pET30a) encodes α_{312} with an S tag and a His_6 tag at the N terminus, and a His_6 tag at the C terminus.

The *Escherichia coli* strain BL21(DE3) was transformed with the α_{312}-pET30a construct. Cells were grown to the mid-log phase and were then induced to express α_{312} by incubating them for 3 h at 37°C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted by centrifugation at 10,000 x *g* for 10 min at 4°C, and then lysed by freeze thawing and sonication in 50 mM NaH_2PO_4 (pH 8 at 4°C), 500 mM NaCl, 10% (w/v) glycerol, 0.02% Brij-35, 5 mM β-mercaptoethanol, 100 μg/ml lysozyme (Sigma), and protease inhibitors (Sigma). The lysate was cleared by centrifugation at 13,000 x *g* for 30 min, adjusted to pH 8 at 4°C, and then filtered through a 0.45-μm filter.

Recombinant α_{312} was purified using a Biologic HR chromatography system (Bio-Rad). All of the purification procedures were conducted at 4°C. The cleared lysate was loaded onto a Ni^{2+} chelating Sepharose column (Amersham Biosciences) that was equilibrated in buffer A (50 mM NaH_2PO_4 [pH 8 at 4°C], 10% w/v glycerol, 0.02% Brij-35 and 500 mM NaCl). The column was washed with 5 column volumes of buffer A, and α_{312} was eluted using a step gradient of imidazole. Fractions that contained purified α_{312} were pooled and exchanged into buffer B (50 mM Tris [pH 8 at 4°C], 100 mM NaCl, 10% (w/v) glycerol, 0.02% (w/v) Brij-35, and 5 mM β-mercaptoethanol) by dialysis. Purified α_{312} was concentrated by centrifugation using Amicon Ultra (Millipore) centrifugal filters with a molecular mass cutoff of 10 kDa. Protease inhibitors (Sigma) and 1 mM DTT were added to the purified α_{312}, which was then stored in aliquots at -80°C. α_{312} was thawed immediately prior to use in the AMPKK assay [16].

AMPKK assay

AMPKK activity was determined by measuring α_{312} activation. The kinase buffer contained 20 mM Tris (pH 8), 50 mM NaCl, 1 mM EDTA, 0.02% (w/v) tween-20, 1 mM DTT, 100 μM ATP, 10 mM $MgCl_2$, a mixture of serine/threonine phosphatase inhibitors (Sigma), a tyrosine phosphatase inhibitor mixture (Sigma), a protease inhibitor mixture (Sigma), and 5 μg α_{312}, unless otherwise indicated. Reactions were initiated by the addition of a sample (3 μg) that had been incubated in kinase buffer with or without α_{312}, for 60 min. Samples were diluted with Laemmli buffer, subjected to SDS-PAGE, and immunoblotted with anti-$pThr^{172}$ AMPK and pan-α AMPK antibodies.

Statistics

Results were analyzed using Student's *t* test and are presented as mean±SEM. Statistical significance was set at $P<0.05$.

Results

High energy phosphates in cardiomyocytes under hypoxic or normoxic conditions

Cardiomyocytes were placed under hypoxic conditions (1% O_2) for 5min, 30min, 1h and 6h. The normoxia group was incubated under standard cell culture conditions (95% air/5% CO_2) and used as a control. Intracellular AMP and ATP levels were determined by HPLC and used to calculate AMP:ATP ratios. Hypoxia is known to increase intracellular AMP and decrease ATP levels due to the inhibition of oxidative phosphorylation in mitochondria. Our results showed that, after 1h under hypoxic conditions, neither intracellular AMP levels nor AMP:ATP ratios had

Table 1. Intracellular AMP and ATP levels in cardiomyocytes under hypoxic and normoxic conditions

	ATP (nmol/mg protein)	AMP (nmol/mg protein)	AMP/ATP
Normoxia	36.71±8.88	0.55±0.13	0.014±0.004
Hypoxia 5min	35.87±6.75	0.58±0.034	0.015±0.003
Hypoxia 30min	35.68±7.81	0.51±0.052	0.014±0.003
Hypoxia 1h	36.83±7.61	0.61±0.044	0.017±0.004
Hypoxia 6h	25.61±7.98#	0.98±0.12#	0.039±0.003#

Rat cardiomyocytes were cultured under hypoxic conditions (1% O2, 9% N2, 5% CO2) for 5min, 30min, 1h and 6h. Rat cardiomyocytes cultured under normoxic conditions (standard cell culture conditions: 95% air, 5% CO2) were used as a control. Intracellular AMP and ATP levels were determined by HPLC and used to calculate AMP/ATP ratios. (Values represent mean±SE for at least three independent assays in duplicate.). #P < 0.01 vs. normoxia.

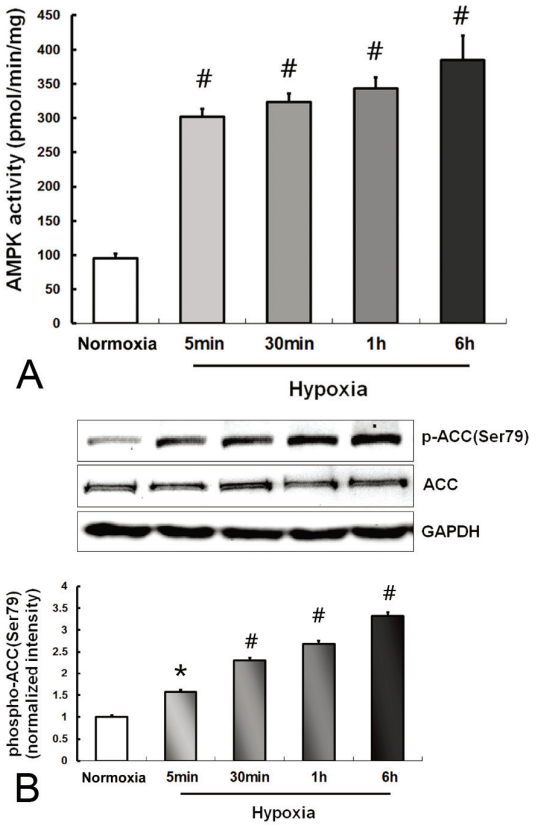

Figure 1. A: AMPK activity of cardiomyocytes under normoxic and hypoxic conditions. (Values represent mean±SE for three independent assays in duplicate). #, P < 0.01 vs. normoxia. B: Western blot analysis showing ACC phosphorylation at Ser^{79} in aerobic and anoxic cardiomyocytes. 40 μg of protein were loaded into each lane. (Values represent mean±SE for three independent assays in duplicate). *, P < 0.05; #, P < 0.01 vs. normoxia.

significantly increased; however, 6h of hypoxia markedly increased both of these parameters (p<0.01 compared with the normoxia group; **Table 1**). These results demonstrate that one hour of hypoxia is not sufficient to increase the conventional parameters associated with increased AMPK activity, including AMP levels and the ratio of AMP to ATP.

AMPK activity and ACC phosphorylation following hypoxia

We also conducted an evaluation of the effects of hypoxia on AMPK activity in cardiomyocytes using SAMS peptide phosphorylation assay. AMPK activity was markedly increased in anoxic cardiomyocytes compared with aerobic cardiomyocytes (p<0.01; **Figure 1A**). This change in AMPK activity was very rapid; it increased by 3-fold 5 minutes after the onset of hypoxia and kept increasing over the entire course of the experiment, although there was no significant difference between activation levels at 5min and at 6h. Since Acetyl-CoA carboxylase (ACC) is a putative downstream target of AMPK [24], we assessed the phosphorylation state of ACC at Ser^{79} by Western blot analysis (**Figure 1B**). ACC phosphorylation was significantly increased in anoxic cardiomyocytes compared with aerobic cells (P<0.05-0.01), while the total abundance of ACC protein remained similar between the two groups (control, 1; 5min, 1.05±0.12; 30min, 1.14±0.27; 1h, 1.12±0.28; 6h, 1.10±0.25). Furthermore, p-ACC was markedly increased as early as 5min (P<0.05), which parallels the increase in AMPK activity observed in anoxic cardiomyocytes.

Phosphorylation of AMPK Thr-172 following hypoxia

An important mechanism that governs the catalytic activity of AMPK is the phosphorylation of the key residue Thr-172 [17]. As our results show, exposure to hypoxia led to a significant

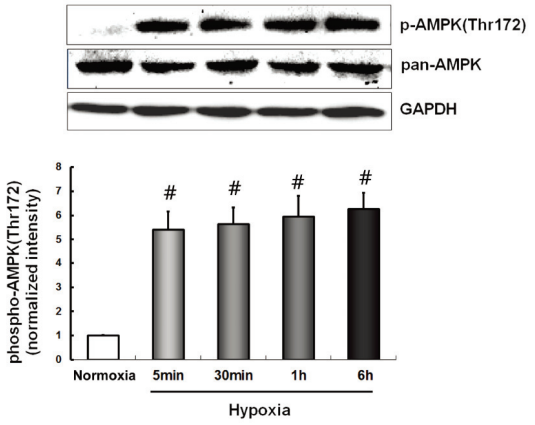

Figure 2. Cardiomyocytes were cultured under hypoxic conditions and cell lysates were subjected to western blot analysis with anti-phosphorylated-AMPK and anti-pan-AMPKα antibodies. Representative immunoblots were used to measure Thr172 phosphorylation on the α-subunit of AMPK. 40 μg of protein were loaded into each lane. (Values represent mean±SE for three independent assays in duplicate). #, P<0.01 vs. normoxia.

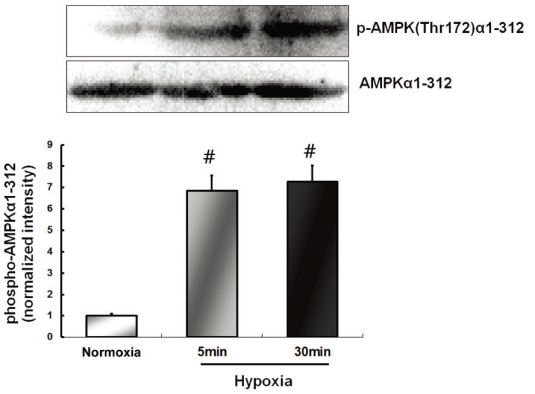

Figure 3. A phospho-Thr-172 AMPKα_{312} immunoblot following a kinase assay of cardiomyocyte lysates cultured under hypoxic conditions for 5 and 15min was incubated with recombinant α_{312} for 60 min. (Values represent mean±SE for three independent assays in duplicate). #, P<0.01 vs. normoxia.

increase in AMPK phosphorylation whithout affecting total AMPKα protein levels between aerobic and anoxic cardiomyocytes (control 1; 5min 0.92±0.11; 30min 1.03±0.08; 1h 0.96±0.10; 6h 0.97±0.14). After 5min of hypoxia, endogenous AMPK Thr-172 phosphorylation (**Figure 2**) was markedly increased, 3- to 4-fold greater than in the normoxia group (P<0.01). As hypoxia was prolonged, relative p-AMPK levels kept increasing, but there was no significant difference between any of the time points. These findings show that AMPK phosphorylation increases critically right at the very early stage of hypoxia onset (**Figure 2**).

AMPKK activity of cardiomyocytes during acute hypoxia

A phospho-Thr-172 AMPKα_{312} immunoblot following a kinase assay of cardiomyocyte lysates cultured under hypoxic conditions for 5 and 15min was incubated with recombinant α_{312} for 30 min. Incubation of the lysates with AMPKα_{312} as a substrate demonstrated a significant (P<0.01) increase in AMPKK activity in hypoxic cardiomyocytes (**Figure 3**).

Discussion

AMPK can be activated in two ways: by allosteric binding of AMP [18], or by phosphorylation by one or more upstream AMPK kinases [19]. Once activated, AMPK minimizes ATP consumption by suppressing ATP-consuming anabolic pathways as well as activating ATP-generating catabolic pathways.

As its name suggests, mammalian AMPK is regulated by changes in the ratio of AMP to ATP. Conditions such as hypoxia lead to the activation of AMPK due to a failure to generate sufficient ATP for cellular functions [20-22]. Thus, under hypoxic conditions, AMPK initiates various adaptive responses in response to changing cellular parameters, namely the decrease in ATP levels or the increase in the AMP: ATP ratio.

However, recent findings challenge the notion that allosteric activation triggered by increased AMP:ATP ratios is the main pathway leading to AMPK activation under hypoxic condition in heart tissues [23]. Herein, we investigated intracellular AMP and ATP concentrations in cardiomyocytes after various durations of hypoxia. Our results demonstrated that, compared with cells cultured under normoxic conditions, neither intracellular AMP levels nor AMP/ATP ratios increased significantly within 1h of hypoxia onset. In contrast, a SAMS peptide phosphorylation assay and immunoblot analysis revealed significant increases in both AMPK activity and ACC phosphorylation within 1h of hypoxic treatment. ACC is a prototypical and well-characterized AMPK target in the heart [24]. One of the ways by which AMPK stimulates ATP synthesis in the heart is by phosphorylating ACC. This phosphorylation inhibits the activity

of ACC, leading to reduced malonyl-CoA formation and relieved inhibition of carnitine palmitoyl transferase 1, ultimately resulting in accelerated β-oxidation of fatty acids and the generation of ATP. These results indicate that, at a very early stage of hypoxia onset in cardiomyocytes, AMPK activity increases independent of AMP concentrations or of the AMP:ATP ratio.

The mechanisms that regulate the increase in AMPKK activity in anoxic cardiomyocytes were elucidated through the use of α_{312} as a substrate to measure AMPK phosphorylation at Thr-172. Reversible phosphorylation of the Thr-172 residue, which is situated within the activation loop of the kinase domain of the AMPK α-subunit, governs the catalytic activity of AMPK [25]. Our results showed that within 5min of exposing cardiomyocytes to hypoxic conditions, AMPK phosphorylation levels were significantly increased, by 3- to 4-fold compared with normoxic controls ($P<0.01$), whereas total AMPKα protein levels did not differ between aerobic and anoxic cardiomyocytes. As hypoxia persisted, relative p-AMPK levels kept increasing, but were not significantly different between any of the time points, suggesting that AMPK phosphorylation is critically increased as of the very earliest stages of hypoxia. The fusion protein [26] was an effective substrate for the in vitro AMPKK assay; our results revealed that incubating cardiomyocyte lysates (under hypoxic conditions for 5min and 15min) with AMPKα_{312} significantly increased AMPKK activity ($P<0.01$). Measuring AMPKK activity in the absence of AMP demonstrated the intrinsic activation of AMPKK in hypoxic cardiomyocytes. Taken together, these results thus demonstrate that AMPKK activity in cardiomyocytes is markedly increased at the very early stage of hypoxia, consistent with the increase in AMPK activity observed under the same conditions. AMPK activation in the absence of measurable changes in AMP concentrations has been implicated in the response of non-cardiac tissues to leptin [27], osmotic stress [28] and metformin [29], but AMPKK activity has not been assessed in these experiments and the specific mediators of presumed AMPKK activation in these settings remain unknown.

In summary, our results demonstrate that in the early stage of hypoxia in cardiomyocytes, increases in AMPK activity occur prior to, and thus independently of, increases in AMP concentration or in the AMP:ATP ratio. Therefore, in these circumstances AMPK is primarily activated by phosphorylation of the conserved Thr-172 residue in its activation loop by its upstream kinase AMPKK.

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Conflict of interest statement

The authors report no conflict of interest.

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