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Hypoxic activation of AMPK is dependent on mitochondrial ROS but independent of an increase in AMP/ATP ratio

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Abstract

AMP-activated protein kinase (AMPK) is a sensor of cellular energy status found in metazoans that is known to be activated by stimuli that increase the cellular AMP/ATP ratio. Full activation of AMPK requires specific phosphorylation within the activation loop of the catalytic domain of the α -subunit by upstream kinases such as the serine/threonine protein kinase LKB1. Here we show that hypoxia activates AMPK through LKB1 without an increase in the AMP/ATP ratio. Hypoxia increased reactive oxygen species (ROS) levels and the antioxidant EUK-134 abolished the hypoxic activation of AMPK. Cells deficient in mitochondrial DNA (p^0 cells) failed to activate AMPK during hypoxia but are able to in the presence of exogenous H_2O_2 . Furthermore, we provide genetic evidence that ROS generated within the mitochondrial electron transport chain and not oxidative phosphorylation is required for hypoxic activation of AMPK. Collectively, these data indicate that oxidative stress and not an increase in the AMP/ATP ratio is required for hypoxic activation of AMPK.

Keywords

AMP-activated kinase; Hypoxia; LKB1; Mitochondria; Reactive oxygen species; Free radicals

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase complex consisting of a catalytic α subunit and two regulatory β and γ subunits [1-3]. AMPK is ubiquitously expressed and functions as an intracellular energy sensor by facilitating ATP production and suppressing unnecessary ATP use in energy-stressed cells. To date conditions that elevate intracellular AMP or decrease ATP levels are known to activate AMPK through the allosteric binding of AMP, which allows AMPK to sense cellular [AMP]/[ATP] ratios. Full activation of AMPK requires specific phosphorylation (Thr172) within the activation loop of the catalytic domain of the α -subunit by upstream kinases, including LKB1, a serine/threonine protein kinase and tumor suppressor [4,5]. Recently, mammalian Ca^{2+} /calmodulin-dependent kinase kinase (CaMKK) has also been identified as an AMPK kinase [6]. AMPK phosphorylates diverse targets, many of which are directly involved in controlling cellular energy metabolism, such as acetyl-CoA carboxylase (ACC) and glycogen synthase [7,8]. The net effect of AMPK activation is to inhibit lipid and glycogen synthesis concomitant with the activation of fatty

acid oxidation and glycolysis. Thus, AMPK switches the cell from energy-storing to releasing energy for use under conditions where ATP is limiting.

Reactive oxygen species (ROS) have been viewed traditionally as damaging to the cell, however, ROS can also function as important activators of key cellular processes, and have been shown to play a vital role in cell signaling networks. For example, the ROS generated within complex III of the mitochondria during hypoxia are both sufficient and required to activate the hypoxia inducible factor-1 (HIF-1) [9-13]. HIF-1 is the key transcription factor that regulates the cellular responses to hypoxia and is critically important for tumor progression and angiogenesis. Hypoxia in the range of 2-20 TORR or 0.3%-5% O₂ in mammalian cells does not result in a change in bioenergetics or induce cell death [14]. By contrast, anoxia is considered 0-2 TORR or 0-0.3% O₂ and results in a decrease in ATP and induction of cell death (Schroedl et al. 2002). However, multiple reports have indicated that hypoxia indeed activates AMPK [15-19]. Here we show that AMPK activation under hypoxic conditions (1.5% O₂) is not associated with an increase in AMP levels, but is dependent on the generation of mitochondrial ROS (mtROS). Furthermore, by using cells deficient in the mitochondrial complex III subunit, cytochrome *b*, we provide evidence here that the mitochondrial ROS, independent, of oxidative phosphorylation is required for the hypoxic activation of AMPK signaling.

Materials and methods

Cell Culture and Reagents

Ampka WT and *Ampka*^{1-/-2-/-} mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% heat inactivated fetal bovine serum (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 20 mM HEPES. WT 143B osteosarcoma cells were cultured as above, whereas the *rho*⁰ (ρ⁰)-143B cells, WT cybrids, and Δ Cytochrome b cybrids were supplemented with 100 µg/ml of Uridine (Sigma). ρ⁰ cells are generated by culturing cells in ethidium bromide at low concentrations (25-50 ng/ml). WT and Δ Cytochrome b cybrids, provided by Carlos Moraes, were previously described [20]. *Lkb1*^{-/-} cells, obtained from R. Depinho and N. Bardeesy, were retrovirally infected with an *Lkb1* cDNA or vector control. These cells were selected in DMEM containing puromycin. All cells were cultured at 37 °C in 5% CO₂ humidified incubators for normoxia conditions. The following reagents were used: AICA-Riboside (AICAR) (Calbiochem), Hydrogen peroxide (Sigma), and EUK-134 (Eukarion, Inc.).

Oxygen conditions

Hypoxic conditions (1.5% O₂ and 5% CO₂ balanced with N₂) were achieved in a humidified variable aerobic workstation (INVIVO O₂, BioTrace), which contains an oxygen sensor that continuously monitors the chamber oxygen tension.

Immunoblot Analysis

Protein levels were analyzed in whole cell lysates obtained using lysis buffer (Cell Signaling) and 50 µg of samples were resolved on a SDS polyacrylamide gel. Gels were then analyzed by immunoblotting with the following antibodies: phospho-Acetyl CoA Carboxylase (Ser79), phospho-AMPKα (Thr172), Acetyl CoA Carboxylase (ACC), and AMPKα (Cell Signaling Technology, Inc.). A representative blot is shown of three independent experiments.

Measurement of AMP, ADP, and ATP

AMP, ADP, ATP measured using HPLC as previously described [21]. Cells were lysed in 300 μ l of media with 20 μ l of 1 M HClO₄. HClO₄ was removed by mixed phase extraction employing 11.75:13.25 (vol/vol) of tri-n-octylamine and Freon 11. Lysates were run on a Zorbax Rx C8 column. The data presented are the results from three independent experiments and the error bars indicate the SEM.

Measurement of ROS

MEFs were infected with 100pfu adenovirus encoding a redox sensitive GFP targeted to the cytosol. Cells were harvested for analysis with the CyanADP Flow Cytometry Analyzer (Dako) 24 or 48 hours after being placed in conditions. The mean fluorescent channel for the ratio of violet excitable to blue excitable was determined with Summit v4.2 software (Dako). The percent oxidized probe is determined as the ratio of the sample mean subtracted from the mean from the probe reduced by 1 mM DTT to the mean from probe oxidized by 1 mM H₂O₂ subtracted from the mean from the probe reduced by 1 mM DTT. Intracellular ROS was also measured using Amplex Red (Molecular Probes) according to manufacturer's protocol. Briefly, cells were lysed in Amplex Red solution (100 μ M) supplemented with horseradish peroxidase (HRP, 2 mUnits/ml) and 200 mUnits/ml of superoxide dismutase (SOD, OXIS International) and incubated in the dark for 30 minutes. Fluorescence was measured in the Spectra Max Gemini plate reader with excitation of 540 nm and emission of 590 nm. The data presented are the result of four independent experiments and the error bars indicate the SEM.

Statistical Analysis

Data are presented as means $\pm$ standard error of mean. One-way analysis of variance was performed to determine the presence of significant differences in the data. When analysis of variance indicated that a significant difference was present, two-sample Student's t-tests were performed to compare experimental data with data gathered at 21% O₂. Statistical significance was determined at a value of $P < 0.05$.

Results and Discussion

In certain oxygen sensitive tissues, it has been proposed that hypoxia inhibits oxidative phosphorylation resulting in an increase in [AMP]/[ATP] ratio and AMPK activation [22]. However, in most tissues mitochondrial respiration is not limited at oxygen levels above 0.3% [23]. Thus, hypoxia in the range of 1-2% O₂ does not inhibit mitochondrial respiration nor increase [AMP]/[ATP] ratio. To assess the levels of ATP, ADP and AMP in wild-type mouse embryonic fibroblasts (MEFs) exposed to normoxia or hypoxia we utilized high-performance liquid chromatography (HPLC). Figs. 1A and C demonstrate that when cells are exposed to hypoxia (1.5% O₂) for 10 or 60 minutes, there were no appreciable changes in AMP, ADP, or ATP levels. By contrast cells exposed to anoxia (0% O₂) show a decrease in ATP levels. Furthermore, only cells exposed to anoxia show an increase in the [AMP]/[ATP] ratio (Figs. 1B and D). These data are consistent with previous investigators who have demonstrated no changes in ATP, ADP and AMP in cells acutely exposed to hypoxia [14]. To determine whether hypoxia activates AMPK, wild-type cells (*Ampk α WT*) and cells deficient in both AMPK α 1 and α 2 (*Ampk α 1^{-/-}2^{-/-}*) were exposed to 21% O₂ (0') or 1.5% O₂ for 30 minutes. Lysates were immunoblotted using an antibody that recognizes the phosphorylation site (Ser79) on acetyl-CoA carboxylase (ACC), a direct target of AMPK [2,24] (Fig. 2A). Hypoxia activates AMPK, as assessed by the phosphorylation of ACC, in *Ampk α WT* cells (Fig. 2A). H₂O₂ treatment also did not activate ACC in the *Ampk α 1^{-/-}2^{-/-}* cells. These data demonstrate that hypoxia activates AMPK signaling independent of an increase in the [AMP]/[ATP] ratio.

The tumor suppressor LKB1 has been identified as the predominant upstream kinase responsible for the phosphorylation of the critical Thr172 site on the α -subunit of AMPK. To determine if LKB1 is the upstream regulator of AMPK under hypoxic conditions, we used *Lkb1*^{-/-} cells that we stably reconstituted with Lkb1 or with a vector control. The reintroduction of Lkb1 rescued AMPK activation under hypoxic conditions. In the *Lkb1*^{-/-} cells reconstituted with the vector control, there was no detectable AMPK activation when exposed to hypoxia for 30 minutes (Fig. 2B). H₂O₂ can slightly activate AMPK in the absence of LKB1 most likely through CaMKK [25-27]. Thus, oxidative stress is likely to activate AMPK through LKB1 dependent and independent signaling pathways.

To determine whether ROS are required for the hypoxic activation of AMPK, *Ampka* WT cells were exposed to 21% O₂ (0'), 1.5% O₂ for 30 minutes or one hour (30' or 60'), or AICA-Riboside (AICAR) (A) in the absence or presence of EUK-134 (10 μ M). AICAR mimics AMP, thereby acting as an activator of AMPK. EUK-134 is a synthetic superoxide dismutase/catalase mimetic, which scavenges both superoxide and H₂O₂. It has been widely used in mammalian models of inflammation and in longevity studies in *C. elegans* [28,29]. As demonstrated in Fig. 3A, EUK-134 prevents the hypoxic activation of AMPK, but has no effect on the AICAR-induced activation of AMPK. Indeed, as detected by a redox sensitive GFP probe, hypoxic generation of ROS can be scavenged by treatment with EUK-134 (Fig. 3B). The redox sensitive GFP probe contains GFP mutations with two oxidant sensitive surface-exposed cysteine residues placed at positions 147 and 204 (S147C and Q204C) [30]. These results were corroborated by using Amplex Red (Fig. 3C). Together these results illustrate that the hypoxic activation of AMPK is dependent on ROS.

To test whether mitochondrial ROS (mtROS) are required for the hypoxic activation of AMPK we generated ρ^0 143B cells. ρ^0 cells are cells depleted of their mitochondrial DNA and are unable to carry out functional electron transport and generate mtROS [31]. WT 143B and ρ^0 143B cells were subjected to 21% O₂ (0'), 1.5% O₂ (30' or 60'), or to H₂O₂ (100 μ M) for 15 minutes. In contrast to WT 143B cells, ρ^0 143B cells fail to activate AMPK under hypoxia, as shown by the phosphorylation of ACC (Figs. 4A and B). As expected both cell types can activate AMPK in response to H₂O₂. The ρ^0 143B cells also failed to increase intracellular hydrogen peroxide levels as determined by Amplex Red (Fig. 4C).

To further explore the role of mtROS in the hypoxic activation of AMPK we used cells that are deficient in cytochrome *b*, a subunit of the mitochondrial complex III. These cells are cybrids that were generated by repopulating ρ^0 143B cells with mitochondria that contain either wild-type (WT) mitochondria DNA or a 4-base pair deletion of the mitochondrial encoded cytochrome *b* gene (Δ Cytochrome *b*) [20]. Δ Cytochrome *b* cells are cytochrome *b* deficient cells that do not consume oxygen, similar to the ρ^0 cells, yet can generate ROS at the Qo site of complex III [32]. Interestingly, hypoxia does activate AMPK in Δ Cytochrome *b* cells (Fig. 5A). These data indicate that the ability of cells to conduct oxidative phosphorylation is not related to their ability to activate AMPK signaling. Furthermore, we show that the activation of AMPK can be inhibited in Δ Cytochrome *b* cells by treatment with EUK-134 (Fig. 5B). The hypoxia-induced increase in intracellular hydrogen peroxide levels could be abrogated by EUK-134 in the Δ Cytochrome *b* cells (Fig. 5C). Collectively, our data indicate that the ROS generated by complex III of the mitochondria is the stimulus for the activation of AMPK signaling and not the rise in AMP levels under hypoxic conditions, thereby identifying a novel signaling pathway for the activation of AMPK.

Our results imply that AMPK can be activated by oxidative stress during hypoxia without an increase in [AMP]/[ATP] ratio. Previous studies have indicated that H₂O₂ increases [AMP]/[ATP] ratio and activates AMPK [33]. To test whether oxidative stress can activate AMPK independently of a change in [AMP]/[ATP] ratio, we exposed WT and ρ^0 143B cells to 20

uM H₂O₂, a smaller concentration than utilized in the literature. Both cell types robustly activated AMPK within 5 minutes (Fig. 6A and B). However, only the WT cells displayed an increase [AMP]/ [ATP] ratio after 5 minutes of 20 uM H₂O₂ (Fig. 6C). The p⁰ cells did not display an increase in [AMP]/ [ATP] ratio, implying that H₂O₂ increases [AMP]/ [ATP] ratio by inhibiting mitochondrial oxidative phosphorylation. These data further indicate that oxidative stress can activate AMPK without increasing the [AMP]/ [ATP] ratio. Mitochondrial oxidative stress is associated but not limited to aging, cancer and diabetes. Our study suggests that mitochondrial oxidative stress might activate AMPK without altering the [AMP]/ [ATP] ratio in these disease processes.

These findings indicate that mitochondrial generated ROS can serve as signaling molecules to activate AMPK. We and others have previously shown that complex III generated ROS are required for hypoxic activation of HIF-1 [9-13]. However, AMPK activation during hypoxia is not required for HIF-1 [16]. The exact role of AMPK activation during hypoxia is not fully understood. Hypoxic activation of AMPK can suppress mTOR [19]. A consequence of mTOR inhibition is the induction of autophagy. Indeed, hypoxia-induced autophagy requires AMPK activation [34]. Thus, AMPK activation during hypoxia is likely an adaptive response to activate autophagy as an early signal to cells that likely will be deprived of nutrients. We suggest that as oxygen levels decrease the mitochondrial complex III acts as an oxygen sensor by releasing ROS into the intermembrane space. Subsequently, the ROS diffuse to the cytosol where they activate a variety of signaling pathways resulting in divergent biological responses such as activation of HIF-1 or AMPK.

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Abbreviations

AICAR	AICA-Riboside
ROS	Reactive Oxygen Species

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Fig. 1.

Hypoxic activation of AMPK is independent of AMP levels. (A) AMP, ADP, and ATP levels were assessed by HPLC in MEFs exposed to 21% O₂ or 1.5% O₂ or 0% O₂ for 10 minutes. (B) AMP/ATP ratio in MEFs exposed to 21% O₂ or 1.5% O₂ or 0% O₂ for 10 minutes. (C) AMP, ADP, and ATP levels were assessed by HPLC in MEFs exposed to 21% O₂ or 1.5% O₂ or 0% O₂ for 60 minutes. (D) AMP/ATP ratio in MEFs exposed to 21% O₂ or 1.5% O₂ or 0% O₂ for 60 minutes.

Fig. 2.

LKB1 is required for the hypoxic activation of AMPK. (A) AMPK activation assessed by phospho-ACC protein in *Ampka* WT and *Ampka1^{-/-}2^{-/-}* cells exposed to 21% O₂ or 1.5%O₂ for 30 minutes, or to 100 μMH₂O₂ for 15 minutes. An anti-ACC antibody was used as loading control. (B) *Lkb1^{-/-}* cells were stably reconstituted with either vector alone or with Lkb1. AMPK activation was then assessed by phospho-ACC protein in both cell types exposed to 21%O₂ or 1.5%O₂ for 30 minutes, or to 100 μMH₂O₂ for 15 minutes. An anti-ACC antibody was used as loading control.

Fig. 3.

ROS is required for the hypoxic activation of AMPK. (A) *Ampka* WT cells were exposed to 21% O₂ (0'), 1.5% O₂ for 30 minutes and 60 minutes, or to 2 mM AICAR for 15 minutes at 21% O₂ ± EUK-134 (10 μM). EUK-134 is a synthetic superoxide dismutase/ catalase mimetic, which scavenges both superoxide and H₂O₂. AMPK activation was then assessed by phospho-ACC protein and an anti-ACC antibody was used as loading control. (B) *Ampka* WT cells were exposed to 21% O₂ (0'), or 1.5% O₂ for 60 minutes ± EUK-134 (10 μM) and ROS was measured using redox sensitive GFP. (C) *Ampka* WT cells were exposed to 21% O₂ (0'), or 1.5% O₂ for 60 minutes ± EUK-134 (10 μM) and ROS was measured using Amplex Red.

Fig. 4.

Hypoxic activation of AMPK requires mitochondrial ROS. (A) WT 143B were exposed to 21% O₂ (0'), to 1.5% O₂ for 30 and 60 minutes (30' or 60'), or to a bolus of H₂O₂ (100 μ M) for 15 minutes. H₂O₂ is used here as a positive control for AMPK activation. An anti-ACC antibody was used as loading control. (B) ρ^0 143B cells were exposed to 21% O₂ (0'), to 1.5% O₂ for 30 and 60 minutes (30' or 60'), or to a bolus of H₂O₂ (100 μ M) for 15 minutes. An anti-ACC antibody was used as loading control. (C) WT 143B and ρ^0 143B cells were exposed to 21% O₂ (0'), or 1.5% O₂ for 60 minutes $\pm$ EUK-134 (10 μ M) and ROS was measured using Amplex Red.

Fig. 5.

Oxidative phosphorylation is not necessary for the hypoxic activation of AMPK. (A) Δ Cytchrome *b* 143B cells were subjected to 21% O₂ (0'), to 1.5% O₂ for 30 and 60 minutes (30' or 60'), or to a bolus of H₂O₂ (100 μ M) for 15 minutes. AMPK activation was then assessed by phospho-ACC protein and an anti-ACC antibody was used as loading control. (B) Δ Cytchrome *b* 143B cells were subjected to 21% O₂ (0') or to 1.5% O₂ for 30 and 60 minutes (30' or 60') $\pm$ EUK-134 (10 μ M). AMPK activation was then assessed by phospho-ACC protein and an anti-ACC antibody was used as loading control. (C) WT 143B and Δ Cytchrome *b* 143B cells were exposed to 21% O₂ (0'), or 1.5% O₂ for 60 minutes $\pm$ EUK-134 (10 μ M) and ROS was measured using Amplex Red.

Fig. 6.

Hydrogen peroxide can activate AMPK without an increase in [AMP]/[ATP] ratio. (A) WT 143B cells and ρ⁰ 143B cells were exposed to 20 μM H₂O₂ for 5 minutes and AMPK activation was measured using the phospho-ACC protein and an anti-ACC antibody was used as loading control. (B) ρ⁰ 143B cells were exposed to 20 μM H₂O₂ for 5 minutes or to AICAR for 30 minutes and AMPK activation was measured using the phospho-AMPK protein and an anti-AMPK antibody was used as loading control. (C) [AMP]/[ATP] ratio in WT 143B cells and ρ⁰ 143B cells exposed to 20 μM H₂O₂ for 5 minutes.