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Oxidative stress activates AMPK in cultured cells primarily by increasing cellular AMP and/or ADP

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ABSTRACT

AMPK is known to be activated by oxidative stress. Addition of glucose oxidase to cells generates H_2O_2 at a constant rate that is opposed by enzymic degradation, providing a good model for physiological oxidative stress. AMPK activation by glucose oxidase correlated with increases in cellular AMP:ATP and was greatly reduced in cells expressing an AMP-insensitive AMPK mutant, although a small degree of activation remained. The effects of increased AMP were partly due to inhibition of Thr172 dephosphorylation. These results suggest that changes in adenine nucleotides, rather than direct oxidative modification, are the major drivers of AMPK activation during oxidative stress.

1. Introduction

The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status, which occurs in all eukaryotes as heterotrimeric complexes comprising catalytic α subunits and regulatory β and γ subunits [1–3]. AMPK is activated by phosphorylation of Thr172 within the kinase domain by upstream kinases, with the principal upstream kinase being the tumor suppressor LKB1 [4–6], and/or by binding of allosteric activators at multiple sites [7]. Binding of AMP to the γ subunit, which is antagonized by ATP, activates AMPK by three complementary mechanisms: (i) allosteric activation; (ii) promotion of Thr172 phosphorylation by LKB1; (iii) inhibition of Thr172 dephosphorylation, which can also be triggered by binding of ADP [8–12]. Cellular stresses that inhibit ATP production or accelerate ATP consumption activate AMPK by causing increases in cellular AMP:ATP and ADP:ATP ratios [8], and AMPK then acts to restore energy homeostasis by switching on catabolic pathways generating ATP, while inhibiting ATP-consuming processes [1–3]. An alternative upstream activating pathway is triggered by increases in cellular Ca^{2+}, causing Thr172 phosphorylation by the calmodulin-dependent protein kinase, CaMKKβ [13–15].

AMPK can also be activated by oxidative stress, usually triggered experimentally by adding reactive oxygen species such as H_2O_2 or NO to the cell medium [16]. Addition of H_2O_2 causes increases in cellular AMP:ATP, suggesting that AMPK activation is via the classical AMP-mediated pathway [17]. To confirm this, we constructed HEK-293 cell lines stably expressing either wild type AMPK (WT cells) or an AMP/ADP-insensitive mutant (RG cells). AMPK was activated by H_2O_2 in WT but not RG cells, while H_2O_2 inhibited oxygen uptake and increased ADP:ATP ratios in both; these results suggest that H_2O_2 activates AMPK by an AMP/ADP-dependent mechanism involving inhibition of the mitochondrial respiratory chain [18]. This was, however, challenged by a recent study in which H_2O_2 was generated in the medium by addition of glucose oxidase (GO); these authors presented evidence for an alternative mechanism involving oxidation of two conserved cysteine residues within the AMPK catalytic subunit [19]. We have therefore re-investigated the mechanism by which oxidative stress activates AMPK.

2. Materials and methods

2.1. Materials and proteins

GO from *Aspergillus niger*, catalase from bovine liver, A23187, H_2O_2 and anti-FLAG antibodies were from Sigma, and STO609 from Tocris Bioscience. A769662 was synthesized in-house [20]. Affinity-purified antibodies against AMPK-α subunits were described

Abbreviations: AMPK, AMP-activated protein kinase; CaMKK, calmodulin-dependent kinase kinase; GO, glucose oxidase; LKB1, liver kinase B1

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previously [21]. Phosphospecific anti-Thr172 antibodies were from Cell Signalling.

2.2. Cell culture

HEK-293 and HeLa cells were from ECACC/HPA (Porton Down, UK) and grown in DMEM containing 4.5 g/L glucose, 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. HEK-293 cells expressing inducible human AMPK-γ2 subunits were generated as follows. DNA encoding full-length γ2 was amplified with primers designed to encode a 5′-BamHI site and a C-terminal FLAG tag, followed by an XhoI site. The resulting PCR product was cloned into the pcDNA5/FRT/TO plasmid (Invitrogen) to create the plasmid pcDND5/FRT/TO/γ2. The R531G mutation was created in this plasmid using the QuikChange Site-Directed Mutagenesis system (Stratagene). T-Rex HEK293 cells containing a single Flp recombinase target (FRT) site (Invitrogen) were transfected with Fugene6 (Promega) using the plasmids POG44 encoding Flp recombinase (Invitrogen) and pcDND5/FRT/TO/γ2 at a ratio of 9:1. After 48 h, cells were detached using trypsin and re-plated in medium containing hygromycin B (200 μg/ml) and blasticidin (15 μg/ml). Medium was replaced every 3 days until cell foci could be identified, and individual foci were then selected and expanded. Expression of AMPK-γ2 (WT or RG) was induced with tetracycline (1 μg/ml) for 48 h.

2.3. AMPK assays in cell lysates

Cell lysates (100 μg protein) were immunoprecipitated by incubation at 4 °C for 2 h on a roller mixer with 6 μl of anti-AMPKα1/α2 antibody coupled to protein G-Sepharose, and the immunoprecipitates assayed for AMPK using the AMARA peptide [22]. When AMPK activity was assayed in HEK-293 cells expressing recombinant FLAG-tagged γ2 subunit, immunoprecipitation was performed using 7 μl of EZview Red anti-FLAG M2 affinity gel from Sigma [18].

2.4. Western blotting and other analytical procedures

For analysis of ACC, SDS–PAGE was performed using Novex NuPAGE Tris–Acetate 3–8% gradient polyacrylamide gels in the Tris–Acetate buffer system. For other proteins, SDS–PAGE was performed using Novex NuPAGE Bis-Tris 4–12% gradient polyacrylamide gels in the MOPS buffer system (Invitrogen). Proteins were transferred to nitrocellulose membranes using the Xcell blot module (Biorad). Membranes were blocked in Li-Cor Odyssey blocking buffer for 1 h and scanned with the Li-Cor Odyssey IR imager using the appropriate secondary antibody coupled to IR680 or IR800 dye. H_2O_2 in cell media was estimated using the Cell Biolabs OxiSelect™ Hydrogen Peroxide Assay Kit. For the estimation of ADP:ATP ratio, cellular nucleotides were extracted in perchloric acid and analysed by capillary electrophoresis [18].

2.5. Statistical analysis

Statistical significance was assessed by 1-way ANOVA using GraphPad Prism 6, with Sidak's multiple comparison test: $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3. Results

3.1. AMPK activation correlates with cell nucleotides when H_2O_2 is generated using glucose oxidase

Pilot experiments revealed that addition of GO at 5 mU/ml or less to HEK-293 cells did not cause significant changes in ADP:ATP ratio, AMPK activity or phosphorylation of the downstream target acetyl-CoA carboxylase (ACC), presumably because any H_2O_2 produced was immediately broken down by cellular enzymes. However, at 10 mU/ml we observed AMPK activation and Thr172 phosphorylation, and a marked phosphorylation of ACC, which were maximal by 20 min and then stable for up to 50 min (Fig. 1A and B). When we estimated the cellular contents of adenine nucleotides, there were decreases in ATP and increases in AMP and ADP that became significant by 20–30 min, which then remained relatively constant up to 50 min (Fig. 1C). As expected [8], the increases in AMP (≈20-fold) were larger than the increases in ADP (3- to 4-fold) or the decreases in ATP (≈2-fold). Thus, AMPK activation and ACC phosphorylation showed a temporal correlation with the increases in AMP and ADP, and the decrease in ATP, during GO treatment.

When we measured H_2O_2 concentration in the medium following addition of 10 mU/ml GO, it increased to 10 μM within 5 min, and then more gradually to around 20 μM by 60 min. By contrast, when a single dose of H_2O_2 was added (to a calculated final concentration of 1 mM), the actual H_2O_2 measured in the medium was only 60 μM at the first time point (2 min), had dropped to <5 μM by 10 min, and was undetectable by 60 min (Fig. 1D). Thus, a single dose of H_2O_2 is metabolized very rapidly by HEK-293 cells, whereas when GO is added a quasi-steady state is reached within 5 min where the rate of H_2O_2 production is balanced by its breakdown.

3.2. Effect of GO in cells expressing an AMP-insensitive AMPK mutant

To test whether AMPK activation by GO was mediated by increases in AMP or ADP, we examined its effects in HEK-293 cells expressing FLAG-tagged AMPK-γ2, either wild type (WT cells) or the AMP/ADP-insensitive R531G mutant (RG cells). These were similar to those used previously [18] except that AMPK-γ2 was expressed from a tetracycline-inducible promoter. When we treated WT cells with GO, there was a large activation (2.5- to 3-fold) of AMPK in anti-FLAG immunoprecipitates between 10 and 20 min that was sustained up to 50 min, similar to the results with endogenous AMPK in Fig. 1. By contrast, there was no activation in RG cells by 20 min, although there was a significant activation at later time points (Fig. 2A).

We also re-examined the effect of adding a single dose of H_2O_2 to the WT and RG cells. Fig. 2B shows the dependence of AMPK activity on H_2O_2 concentration, measured 60 min after addition, while Fig. 2C shows the time course with 1 mM H_2O_2. Note that the H_2O_2 concentrations in Fig. 2B are calculated, although Fig. 1D suggests that the actual concentrations decline very rapidly following addition to cells. Activation of WT AMPK was observed 60 min after addition of H_2O_2 to 300 μM or 1 mM, but not 100 μM (Fig. 2B). As previously reported [18], no activation was observed 60 min after addition with the RG mutant at any H_2O_2 concentration. However, some activation by 1 mM H_2O_2 was seen with the RG mutant after 10 min, which then declined to baseline by 60 min. Maximal activation of AMPK in WT cells were observed at 10 min after which it declined, although >2-fold activation was still observed at 60 min (Fig. 2C).

3.3. Effect of catalase and the CaMKK inhibitor, STO609

To confirm that the effect of GO was mediated by generation of H_2O_2, we pre-treated WT and RG cells with catalase prior to addition of GO or H_2O_2, and then measured AMPK activity and Thr172 phosphorylation 50 min later (Fig. 3A and B). The results showed that catalase alone had no effect, but that it abolished the large increases in AMPK activity and Thr172 phosphorylation observed in response to GO or H_2O_2 in the WT cells, as well as the much smaller increases observed in the RG cells. We also pretreated WT and RG cells with the CaMKK inhibitor STO609 [23], and then

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