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\textsubscript{2}O\textsubscript{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 \( \alpha \) subunits and regulatory \( \beta \) and \( \gamma \) 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 \( \gamma \) 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\textsuperscript{2+}, causing Thr172 phosphorylation by the calmodulin-dependent protein kinase, CaMK\( \text{\textgreek{i}} \) [13–15].

AMPK can also be activated by oxidative stress, usually triggered experimentally by adding reactive oxygen species such as H\textsubscript{2}O\textsubscript{2} or NO to the cell medium [16]. Addition of H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} in WT but not RG cells, while H\textsubscript{2}O\textsubscript{2} inhibited oxygen uptake and increased ADP:ATP ratios in both; these results suggest that H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} and anti-FLAG antibodies were from Sigma, and STO609 from Tocris Bioscience. A769662 was synthesized in-house [20]. Affinity-purified antibodies against AMPK-\( \alpha \) subunits were

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described 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 Xhol 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 QuickChange Site-Directed Mutagenesis system (Stratagene). T-Rex HEK293 cells containing a single Flp recombimase 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/γ2 antibody coupled to protein G-Sepharose, and the immunoprecipitates assayed for AMPK by 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. 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 H2O2 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 H2O2 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 H2O2 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 H2O2 was added (to a calculated ﬁnal concentration of 1 mM), the actual H2O2 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 H2O2 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 H2O2 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 H2O2 to the WT and RG cells. Fig. 2B shows the dependence of AMPK activity on H2O2 concentration, measured 60 min after addition, while Fig. 2C shows the time course with 1 mM H2O2. Note that the H2O2 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 H2O2 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 H2O2 concentration. However, some activation by 1 mM H2O2 was seen with the RG mutant after 10 min, which then declined to baseline by 60 min. Maximal activation of AMPK in WT cells was 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 H2O2, we pre-treated WT and RG cells with catalase prior to addition of GO or H2O2, 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 H2O2 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 incubated with GO or the Ca\(^{2+}\) ionophore A23187 for 50 min. Although STO609 abolished the effect of A23187, it did not affect the response to GO in either WT or RG cells, showing that increased AMPK activity and Thr172 phosphorylation was not dependent on CaMKKube under these conditions.

3.4. \(\text{H}_2\text{O}_2\) treatment inhibits Thr172 dephosphorylation in intact cells

We suspected that the increased Thr172 phosphorylation observed in response to \(\text{H}_2\text{O}_2\) was mediated by binding of AMP to the AMPK-\(\gamma\) subunit, leading to inhibition of Thr172 dephosphorylation. To address this, we switched to HeLa cells. Due to the complete absence of LKB1 in these cells, basal phosphorylation of Thr172 is extremely low, but can be increased by addition of the Ca\(^{2+}\) ionophore A23187, which activates CaMKK\(\beta\) [13]. Pilot experiments revealed that Thr172 phosphorylation had reached a new steady state 30 min after A23187 addition. At that point we added enough STO609 to completely inhibit CaMKK\(\beta\), providing an opportunity to measure the rate of Thr172 dephosphorylation in intact cells. This experimental protocol is summarized in Fig. 4A.

We first examined the effect of \(\text{H}_2\text{O}_2\) and A23187 on adenine nucleotide ratios. Fig. 4B shows that treatment with 1 mM \(\text{H}_2\text{O}_2\) for 10 min increased the cellular ADP:ATP ratio by 7-fold. Interestingly, this was reduced to 3- to 4-fold if AMPK had been activated...
by adding A23187 prior to H$_2$O$_2$, although A23187 had no effect on its own.

In HeLa cells not treated with H$_2$O$_2$, treatment with A23187 for 30 min caused a large AMPK activation and Thr72 phosphorylation, as expected (Fig. 4C, lanes 1 and 2). Interestingly, in cells to which H$_2$O$_2$ had been added 20 min after A23187, a higher steady state level of AMPK activation and Thr72 phosphorylation was observed (compare lanes 2 and 5). When STO609 was added to block CaMKKβ, there was an extremely rapid AMPK inactivation, concomitant with Thr172 dephosphorylation, in cells not pre-treated with H$_2$O$_2$. However, both effects were markedly reduced in the cells pre-treated with H$_2$O$_2$, which was particularly evident in the samples taken 1 min after addition of STO609.

4. Discussion

These results resolve some of the discrepancies between our previous study [18] and that of Zmijewski et al. [19]. We reported that WT AMPK was markedly activated by a single dose of H$_2$O$_2$, whereas there was no activation of the RG mutant, and therefore concluded that the effect of H$_2$O$_2$ was mediated entirely through changes in cellular adenine nucleotides [18]. However, we had used a standard incubation time of 60 min for all AMPK activators examined in our study, and had not realized how rapidly a single dose of H$_2$O$_2$ is metabolized by cells (see Fig. 1D). Our present results (Fig. 2C) show that H$_2$O$_2$ did cause a small but significant activation of the RG mutant after 10 min, but that this had reverted to baseline by 60 min, which is why we previously missed it. The much larger activation observed with WT AMPK also declined between 10 and 60 min but was still evident at 60 min, as reported previously [18]. The reversal of AMPK activation between 10 and 60 min is presumably because the added H$_2$O$_2$ has been almost completely metabolized within 10 min (Fig. 1D), and because cellular anti-oxidant systems are able to partially reverse any oxidative damage within this timeframe.

Zmijewski et al. [19] used the alternative approach of adding GO, which generates H$_2$O$_2$ as a by-product of metabolism of medium glucose. The advantage of this is that, after a brief lag, the rate of production of H$_2$O$_2$ by GO is balanced by its destruction by cellular enzymes, so that a quasi-steady state is reached where the concentration of H$_2$O$_2$ in the medium remains approximately constant (Fig. 1D). This probably represents a better model of physiological oxidative stress than addition of a single high dose of H$_2$O$_2$. After a lag of 10–20 min, GO activated wild type AMPK by up to 5-fold, accompanied by increased phosphorylation of its downstream target ACC, and correlating with increases in AMP and ADP and decreases in ATP (Fig. 1). AMPK activation in response to GO was much lower in RG cells, although there was a small but significant activation after 30 min (Fig. 2A). The activation of the RG mutant we observed after 10 min treatment with a single dose of H$_2$O$_2$
While this study was in progress, a potential mechanism was proposed by which AMPK is inactivated (rather than activated) by oxidative stress in cardiac myocytes [27]. By over-expressing a substrate-trapping mutant of thioredoxin-1 (Trx1) in mouse heart, AMPK-α subunits were identified as major intracellular targets of Trx1. AMPK activation during ischemia was abolished in hearts of transgenic mice expressing Trx1 with mutations in its critical cysteine oxidation mechanism proposed by Zmijewski et al. [19], with oxidative stress promoting ADP-ribosylation of glycer-alddehyde-3-phosphate dehydrogenase [25], both of which would inhibit glycolysis.

Because of the lack of LKB1 and the low basal activity of CaMKKβ, basal Thr172 phosphorylation and AMPK activity in HeLa cells is very low. However, addition of the Ca2+ ionophore A23187 caused AMPK activation and Thr172 phosphorylation, which reached a steady state within 30 min, when Thr172 phosphorylation by CaMKKβ was balanced by dephosphorylation. If the CaMKK inhibitor STO609 was then added, we could directly observe dephosphorylation within the intact cell in the absence of simultaneous phosphorylation. Interestingly, without H2O2 treatment this dephosphorylation appeared to be complete 1 min after STO609 addition, showing that the phosphate on Thr172 was turning over extremely rapidly. However, when H2O2 was added 10 min prior to STO609, an initial increase in steady state AMPK activation and Thr172 phosphorylation was observed, and then the rate of dephosphorylation on STO609 addition was drastically reduced. We suggest that this was primarily due to inhibition of dephosphorylation due to binding of AMP or ADP to the AMPK-γ subunit, which would explain both the increase in steady state AMPK activation/Thr172 phosphorylation prior to STO609 addition, and the reduced rate of inactivation/dephosphorylation following its addition. We cannot, however, rule out the additional possibility that protein phosphatase(s) acting on Thr172 are directly inhibited by oxidative stress.

Interestingly, while H2O2 caused increases in cellular ADP:ATP ratio in HeLa cells, they were significantly smaller in cells pre-treated with A23187 for 20 min (Fig. 4B). This could be explained if AMPK activation by A23187 caused metabolic changes that better prepared the cells for the energy stress caused by subsequent addition of H2O2. AMPK activation has been previously shown to induce expression of target genes for FOXO3, including several proteins involved in resistance to oxidative stress [26]. However, the effect we observed occurred after only 20 min, and changes not requiring transcription and translation, such as acute activation of glucose uptake and glycolysis [2] may perhaps be more likely to explain it.
disulfide, and two conserved cysteine residues in the AMPK-α subunit (Cys130 and Cys174) were identified whose reduction by Trx1 prevented AMPK inactivation during glucose deprivation in cardiac myocytes, or cardiac ischemia in vivo. These cysteines are within the kinase domain (Cys174 is almost adjacent to Thr172) and are distinct from those that Zmijewski et al. [19] suggested to be involved in AMPK activation during oxidative stress. Given the crucial requirement for AMPK in the response to cardiac ischemia, it was suggested that Trx1 was an essential cofactor that protects against oxidative inactivation of AMPK during ischemia [27]. Interestingly, they did not find any evidence for oxidative modification of AMPK when HEK-293 cells were incubated with 300 μM H2O2 for 30 min, although Thr172 phosphorylation increased, presumably due to increased AMP inhibiting dephosphorylation as shown here. The authors speculated that, compared with primary cells, immortalized cell lines may have elevated levels of anti-oxidant defense systems that prevent inactivation of AMPK during oxidative stress, thus allowing mechanisms causing activation to become apparent [27].

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