

Exposure to Hydrogen Peroxide Induces Oxidation and Activation of AMP-activated Protein Kinase^{*[S]}

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Although metabolic conditions associated with an increased AMP/ATP ratio are primary factors in the activation of 5'-adenosine monophosphate-activated protein kinase (AMPK), a number of recent studies have shown that increased intracellular levels of reactive oxygen species can stimulate AMPK activity, even without a decrease in cellular levels of ATP. We found that exposure of recombinant AMPK $\alpha\beta\gamma$ complex or HEK 293 cells to H₂O₂ was associated with increased kinase activity and also resulted in oxidative modification of AMPK, including S-glutathionylation of the AMPK α and AMPK β subunits. In experiments using C-terminal truncation mutants of AMPK α (amino acids 1–312), we found that mutation of cysteine 299 to alanine diminished the ability of H₂O₂ to induce kinase activation, and mutation of cysteine 304 to alanine totally abrogated the enhancing effect of H₂O₂ on kinase activity. Similar to the results obtained with H₂O₂-treated HEK 293 cells, activation and S-glutathionylation of the AMPK α subunit were present in the lungs of acatalasemic mice or mice treated with the catalase inhibitor aminotriazole, conditions in which intracellular steady state levels of H₂O₂ are increased. These results demonstrate that physiologically relevant concentrations of H₂O₂ can activate AMPK through oxidative modification of the AMPK α subunit. The present findings also imply that AMPK activation, in addition to being a response to alterations in intracellular metabolic pathways, is directly influenced by cellular redox status.

AMPK³ is a serine/threonine kinase that consists of three subunits, of which the α subunit has inducible kinase activity

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³ The abbreviations used are: AMPK, 5'-adenosine monophosphate-activated protein kinase; ROS, reactive oxygen species; ATZ, 3-amino-1,2,4-triazole; AID, autoinhibitory domain; GO, glucose oxidase; DCF, 2',7'-dichlorodihydrofluorescein; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; GSS, S-glutathionylation of; BIAM, biotinylated iodoacetamide; ACC, acetyl-CoA carboxylase.

and the β and γ subunits have regulatory function. Formation of the $\alpha\beta\gamma$ complex is required for optimal allosteric activation of AMPK, which is induced by binding of AMP to the γ subunit (1–4). In addition to activation by AMP, phosphorylation of the Thr¹⁷² residue of the α subunit enhances kinase activity (5, 6). Recent studies have shown that the autoinhibitory domain (AID), located between amino acids 312 and 335 of the AMPK α subunit, is responsible for the lack of kinase activity under basal conditions (7–9), whereas AMP-induced conformational changes within the $\alpha\beta\gamma$ complex diminish function of the AID and lead to kinase activation.

The regulation of AMPK activity is primarily thought to result from alterations in the intracellular AMP/ATP ratio, arising from diminished ATP generation due to hypoxia, glucose deprivation, heat shock, or reduction in mitochondrial oxidative phosphorylation or from increased ATP consumption, such as occurs during strenuous exercise (2, 10–12). Once activated, AMPK can phosphorylate and modulate the function of essential metabolic pathways participating in the regulation of glucose and lipid homeostasis (13–15). A major effect of AMPK activation is in preserving energy for use under conditions where ATP is limiting (4, 16). AMPK activation appears to prevent or diminish inflammation-associated organ injury, including the development of atherosclerotic cardiovascular disease in diabetes (17), ischemia-induced cardiac dysfunction (18–20), and hepatic dysfunction in animal models of nonalcoholic steatohepatitis as well as in humans with this condition (21, 22). Our studies have also suggested that therapeutic approaches to increase AMPK activity diminish the severity of LPS-induced acute lung injury in mice (23, 24).

Although increased formation of reactive oxygen species (ROS) is generally thought to be associated with pathophysiological situations leading to cellular injury and organ dysfunction, recent studies have shown beneficial effects of ROS in modulating inflammation, including TLR4-induced neutrophil activation and LPS-associated acute lung injury (24–27). Several studies have demonstrated that increased intracellular concentrations of H₂O₂ result in activation of AMPK and enhancement of AMPK-mediated cellular adaptation (28–30), including maintenance of redox homeostasis (31, 32). In cardiac preconditioning studies, antioxidants diminished H₂O₂-associated activation of AMPK and resulted in increased severity of ischemia-reperfusion-induced cardiac heart injury (33).

Despite the ability of increased intracellular concentrations of H₂O₂ to induce AMPK activation in many cell types, the mechanism for this effect has not been well characterized. Whereas initial reports showed that H₂O₂-dependent activation of AMPK resulted from ATP depletion and increased AMP/ATP ratios (34), other studies demonstrated that increased intracellular concentrations of H₂O₂ were associated with activation of AMPK before or without alteration in the ATP/AMP ratio (35–37).

H₂O₂ can affect redox-sensitive signaling pathways as a result of oxidative modification of cysteine residues in proteins (38–40). We therefore hypothesized that a potential mechanism by which increased intracellular concentrations of H₂O₂ can activate AMPK is through oxidation of cysteines in one or more AMPK subunits. Our present experiments demonstrate that exposure to H₂O₂ is associated with cysteine oxidation in the AMPK α subunit and is able to directly activate AMPK.

EXPERIMENTAL PROCEDURES

Mice—Male C57BL/6, C3HeB/FeJ, or acatalasemic C3Ga.Cg-Cat B/J mice, 8–12 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were kept on a 12 h/12 h light/dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols (University of Alabama at Birmingham Institutional Animal Care and Use Committee).

Acute Lung Injury Model—Acute lung injury was induced by intratracheal administration of 1 mg/kg LPS in 50 μ l of PBS as described previously (23–25, 41). Briefly, mice were anesthetized with isoflurane and then suspended by their upper incisors on a 60° incline board. The tongue was gently extended, and LPS solution was deposited into the pharynx (24, 42, 43). Mice were pretreated with saline or ATZ (500 mg/kg body weight dissolved in 0.9% saline) i.p., and 4 h later LPS (1 mg/kg) was administered intratracheally. Lungs were harvested 24 h after LPS administration.

Culture of Human Embryonic Kidney Cells—HEK 293 cells were maintained at 37 °C in 5% CO₂ in RPMI 1640 growth medium (Invitrogen) that contained 8% fetal bovine serum (Atlanta Biologicals; Norcross, GA), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 ng/ml) (Sigma). Prior to use in experiments, the cells were washed twice and incubated with RPMI 1640 medium (FBS, 0.5%) for 1 h and then treated as described in the figure legends.

Reagents and Antibodies—Purified human AMPK $\alpha\beta\gamma$ complex (>95% purity; specific activity, 737 nmol⁻¹ min⁻¹ mg⁻¹) was obtained from SignalChem (Richmond, Canada). AMPK α , AMPK β , or AMPK γ antibody was purchased from Cell Signaling (Beverly, MA). Biotinylated glutathione ethyl ester and streptavidin-agarose were purchased from Invitrogen, whereas hydrogen peroxide, glutathione, and aminotriazole (ATZ) were obtained from Sigma. Bio-Gel P10 was purchased from Bio-Rad. 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside was purchased from Toronto Research Chemicals (Toronto, Canada).

Measurement of AMPK Activity—AMPK activity was determined using a radiometric assay and SAMS peptide substrate,

as described previously (44) with minor modifications. Briefly, recombinant AMPK $\alpha\beta\gamma$ complex (25 ng/sample) or purified FLAG-AMPK α was incubated in kinase buffer (60 μ l/sample) (MOPS (5 mM), β -glycerol-phosphatase (2.5 mM), MgCl₂ (5 mM), 1 mM EGTA, and diethylene triamine pentaacetic acid (100 μ M)) and SAMS peptide (5 μ g/sample) for 10 min at room temperature. The phosphorylation of SAMS peptide was initiated by the inclusion of 0.2 μ l of [³²P]ATP and cold ATP (20 μ M) mix, and samples were incubated at 30 °C. The reaction mix (2 μ l) was transferred to phosphocellulose P81 at the times indicated in the figure legends. Air-dried phosphocellulose P81 was washed three times (10 min each wash) in phosphoric acid (1%) solution. The phosphocellulose P81 was then subjected to autoradiography, and dot density was determined using Alpha-Innotech software (Santa Clara, CA).

Western Blot Analysis of AMPK Subunits—Lung homogenates or extracts from HEK 293 cells were prepared in lysis buffer (Tris, pH 7.4 (50 mM), NaCl (150 mM), Nonidet P-40 (0.5% v/v), EDTA (1 mM), EGTA (1 mM), Na₃VO₄ (1 mM), NaF (50 mM), and protease inhibitors) and then sonicated and centrifuged at 10,000 \times g for 15 min at 4 °C. The protein concentration in supernatants was determined using Bradford reagent (Bio-Rad) with BSA as a standard (24, 45). Samples were mixed with Laemmli sample buffer and boiled for 5 min. Equal amounts of protein were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Immobilon P, Millipore, Billerica, MA). The membranes were probed with specific antibodies to AMPK subunits, followed by detection with horseradish peroxidase-conjugated anti-mouse or goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (SuperSignal; Pierce). Each experiment was carried out two or more times using HEK 293 cells or with lung homogenates obtained from separate groups of mice.

Imaging of DCF Fluorescence—Intracellular levels of ROS, including H₂O₂, were measured using the redox-sensitive probe DCFH-DA (46) in conjunction with fluorescent microscopy (24, 26, 38, 45, 47, 48). Briefly, HEK 293 cells (~80% confluent) were incubated in a 4-well chambered coverglass (Nalge; Naperville, IL) with DCFH-DA (10 μ M) for 60 min, followed by treatment with H₂O₂ (0 or 200 μ M) or glucose oxidase (10 milliunits/ml) at 37 °C. Images were acquired at the indicated time periods (as described in the figure legends) by single bidirectional scans of live cells using a Leica DMIRBE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT laser confocal optics. The pinhole setting was 0.2 Airy units, and laser excitation was set for 5% to avoid dye photo-oxidation. The levels of fluorescence were averaged using SimplePCI software (Compix, Cranberry Township, PA). Images were processed using IPLab Spectrum and Adobe Photoshop (Adobe Systems) software.

S-Glutathionylation of AMPK—Purified AMPK (100 ng) in kinase buffer containing diethylene triamine pentaacetic acid (100 μ M) was incubated with GSH-biotin (0 or 100 μ M) for 15 min, followed by exposure to H₂O₂ (200 μ M) for an additional 5 min. Samples were then boiled in Laemmli sample buffer (without DTT) for 5 min, resolved in non-reducing SDS-PAGE, followed by Western blot analysis with streptavidin-HRP. Mem-

H₂O₂-induced AMPK Activity

branes were subsequently re probed with antibodies specific for AMPK subunits.

Detection of GSS-AMPK Adduct Formation in HEK 293 Cells—HEK 293 cells (2×10^6 /ml) were incubated with ethyl ester GSH-biotin (6 mM) for 1.5 h. The cells were then washed twice with culture buffer to remove the excess of GSH and treated with H₂O₂ (0 or 300 μ M) for 15 or 30 min. Cell lysates were prepared in the presence of *N*-ethylmaleimide (5 mM) and then passed through Bio-Gel P10 to remove free GSH-biotin and *N*-ethylmaleimide. The level of GSS-protein conjugates was determined using non-reducing Western blot analysis with streptavidin-HRP, whereas GSS-AMPK subunit levels were measured after pull-down with streptavidin-agarose (60 min at 4 °C), followed by reducing SDS-PAGE and Western blot analysis with antibodies to the AMPK α , AMPK β , or AMPK γ subunits.

Labeling of AMPK Free Cysteine Thiols—The extent of free (unoxidized) cysteine residues within AMPK subunits was determined using the BIAM labeling assay (49–52). Briefly, cell extracts or lung extracts (0.4 mg/sample) obtained from control, acatalasemic, or ATZ-treated mice were incubated with BIAM (200 μ M) for 30 min at room temperature, and then excess BIAM was removed by passing the extracts through Bio-Gel P10. Next, BIAM-protein conjugates were precipitated with streptavidin-agarose for 1 h at 4 °C. Samples were washed four times with lysis buffer containing 0.5% SDS to obtain specific pull-down of biotinylated proteins and to avoid potential contamination with unlabeled proteins. BIAM-protein adducts were extracted from streptavidin agarose by boiling in Laemmli sample buffer for 10 min and then subjected to reducing SDS-PAGE and Western blot analysis with antibodies to the AMPK α , AMPK β , or AMPK γ subunits.

Metal-catalyzed Oxidation of Purified AMPK—Human purified AMPK $\alpha\beta\gamma$ complex (25 ng) was incubated with H₂O₂ (0 or 100 μ M) in the presence or absence of Fe²⁺ (100 μ M) or Cu¹⁺ (100 μ M) in kinase buffer (without diethylene triamine pentacetic acid) for 10 min at 25 °C, followed by measurement of AMPK activity over the next 30 min.

Autophosphorylation of AMPK—Human purified recombinant AMPK $\alpha\beta\gamma$ complex (100 ng) was incubated in kinase buffer with H₂O₂ (0, 100, or 200 μ M) for 10 min at room temperature, and then 0.2 μ l of [³²P]ATP and ATP (20 μ M) were added to the cultures for an additional 30 min at 37 °C. Proteins were then subjected to SDS-PAGE and autoradiography.

Co-immunoprecipitation—Cells expressing FLAG-tagged AMPK α (WT) or AMPK α (amino acids 1–335) truncation were lysed in immunoprecipitation buffer (53) that preserves protein-protein interactions, followed by incubation of cell extracts with anti-FLAGM2 beads for 60 min at 4 °C. Beads were washed with immunoprecipitation buffer four times. The amount of AMPK β subunit associated with AMPK α or AMPK α 1–335 was then determined by subsequent probing of the Western blot membrane with anti-FLAG and anti-AMPK β antibodies.

Construction of Expression Plasmids and Recombinant Protein Expression—Full-length human AMPK α cDNA was purchased from Open Biosystems and cloned into 3XFLAG-CMV10 (Sigma) for mammalian expression. FLAG-tagged AMPK α and the C-terminal truncation mutants AMPK α

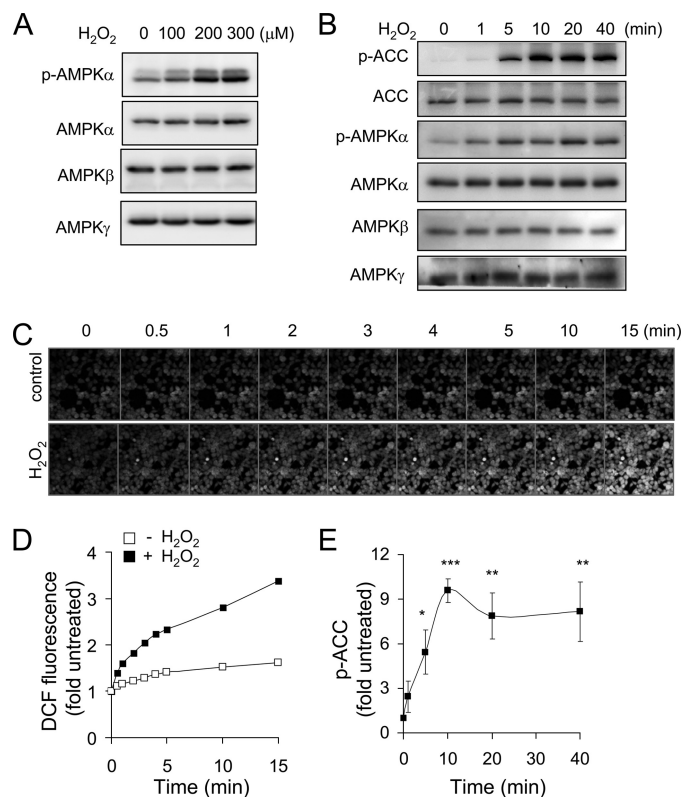


FIGURE 1. Exposure to H₂O₂ induces rapid activation of AMPK and DCFH-DA oxidation in HEK 293 cells. *A* and *B*, representative Western blots show levels of the AMPK α , AMPK β , and AMPK γ subunits, phospho-Thr¹⁷²-AMPK α , ACC, and phospho-Ser⁷⁹-ACC obtained from HEK 293 cells exposed to H₂O₂ (0, 100, 200, or 300 μ M) for 60 min (*B*) or cells treated with H₂O₂ (250 μ M) for 0–40 min (*A*). Confocal images (*C*) and mean DCF fluorescence (*D*) obtained from cells treated with H₂O₂ (250 μ M) for 0–15 min. Confocal images of the same cell populations were acquired at the indicated time period. A second experiment provided similar results. *E*, quantitative analysis of phospho-Ser⁷⁹-ACC (*p*-ACC) in cells treated as described in *A*. Shown is the mean \pm S.D. (error bars), $n = 3$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

1–335 and AMPK α 1–312 were obtained by insertion of PCR products into 3XFLAG-CMV10. Mutation of cysteine 299, 304, or 312 to alanine within FLAG-tagged full-length AMPK α (WT) or truncated AMPK α (1–312) was performed using standard mutagenesis techniques. Transiently expressed FLAG-AMPK α , WT, or truncation mutants were purified using anti-M2 FLAG-agarose beads as described previously (54) and then subjected to Western blot analysis with anti-FLAGM2 or anti-phospho-Thr¹⁷²-AMPK antibodies. In parallel experiments, beads containing FLAG-AMPK α (e.g. WT or truncation mutants) were washed twice with kinase buffer, and AMPK activity was determined using a radiometric assay and SAMS peptide as a substrate.

Measurement of Cellular Nucleotides—The levels of ATP, ADP, and AMP in HEK 293 cells were determined by etheno derivatization and subsequent HPLC analysis of the resulting fluorescent species, as described previously (55, 56).

Statistical Analyses—Experiments with purified AMPK or HEK 293 cells were each performed two or more times. Student's *t* test was used for comparisons between two groups, whereas Tukey's test was performed for comparisons between more than two groups, with $p < 0.05$ considered to be statistically significant. Mouse lung homogenates were obtained from

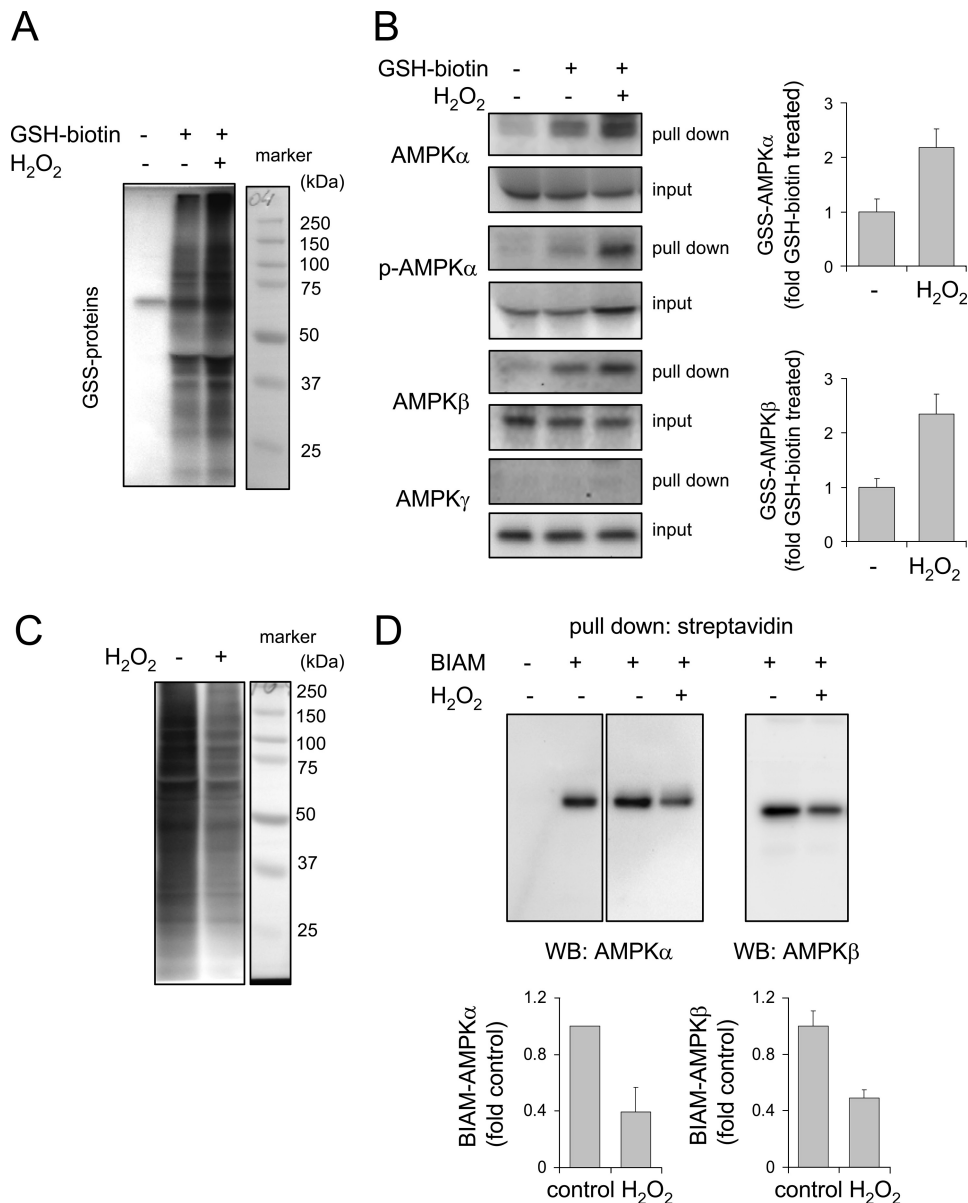


FIGURE 2. H₂O₂ induces oxidation of cysteine residues within AMPK subunits. *A*, HEK 293 cells loaded with or without EE-GSH-biotin were incubated with H₂O₂ (0 or 250 μ M) for 20 min, and the amounts of GSS-protein adduct formation were determined using non-reducing SDS-PAGE and Western blot analysis with streptavidin-HRP. *B*, cell extracts obtained under the same conditions as in *A* were incubated with streptavidin-agarose, and the amounts of GSS-protein conjugates of the AMPK α , AMPK β , and AMPK γ subunits as well as of phospho-Thr¹⁷²-AMPK α (p-AMPK α) were determined by probing the Western blots with specific antibodies. *Right*, mean \pm S.D. (error bars) obtained from two experiments. *C*, cellular proteins were incubated with or without H₂O₂, followed by incubation with BIAM. Western blot analysis with streptavidin-HRP was then performed. *D*, protein-BIAM adducts were purified using streptavidin pull-down and then subjected to Western blot analysis (WB) using anti-AMPK α or AMPK β antibodies (mean \pm S.D. obtained from two experiments).

two separate groups of control or acatalasemic mice or mice treated with ATZ ($n = 3$ mice in each group).

RESULTS

Exposure of HEK 293 Cells to H₂O₂ Results in Activation of AMPK—ROS, including H₂O₂, have been shown to activate AMPK in macrophages, neutrophils, and other cell populations. As shown in Fig. 1, *A* and *B*, exposure of HEK 293 cells to H₂O₂ resulted in activation of AMPK that was dose- and time-dependent (Fig. 1, *A* and *B*). Activation of AMPK occurred rapidly after cellular exposure to H₂O₂, with increased levels of

phospho-Thr¹⁷²-AMPK and phospho-Ser⁷⁹-ACC being detected within ~ 1 –5 min after the addition of H₂O₂ to the cultures. The addition of H₂O₂ to HEK 293 cells resulted in enhanced DCF fluorescence, an indicator of increased intracellular ROS concentrations (Figs. 1, *C* and *D*), within 1 min, suggesting that the increase in the intracellular levels of H₂O₂ was responsible for the activation of AMPK in H₂O₂-exposed HEK 293 cells.

H₂O₂ Induces S-Glutathionylation of AMPK Subunits—Alterations in intracellular concentrations of H₂O₂ can modulate the function of intracellular signaling pathways through oxidative modification, including S-glutathionylation, of cysteine residues (57, 58). To determine if exposure of HEK 293 cells to H₂O₂ had such effects on AMPK, cells loaded with ethyl ester GSH-biotin were incubated with H₂O₂. As shown in Fig. 2*A*, exposure to H₂O₂ resulted in an increase in the levels of GSS-protein adduct formation in HEK 293 cells.

We next determined whether H₂O₂ exposure produced S-glutathionylation of AMPK subunits. In these experiments, GSS-protein conjugates were precipitated using streptavidin-agarose, followed by Western blot analysis of AMPK subunits. As shown in Fig. 2*B*, there were increased amounts of GSS-phospho-Thr¹⁷²-AMPK α and GSS-AMPK β , but not of GSS-AMPK γ , in cells treated with H₂O₂. Similarly, increased oxidation of cysteine residues within the AMPK α and AMPK β subunits was detected when cell extracts were incubated with BIAM and H₂O₂. Under these

conditions, a decrease in BIAM-protein adduct formation, such as was found for the AMPK α and AMPK β subunits after direct exposure to H₂O₂, indicated the presence of oxidized cysteine residues that were unable to react with BIAM (Fig. 2, *C* and *D*). These results show that activation of AMPK by H₂O₂ is associated with enhanced oxidative modification of both the AMPK α and AMPK β subunits.

Activation of AMPK as Well as Cysteine Oxidation in the AMPK α Subunit Precede Decline in ATP Levels in H₂O₂-exposed HEK 293 Cells—Although the above experiments demonstrated that cellular exposure to H₂O₂ resulted in activation as

H₂O₂-induced AMPK Activity

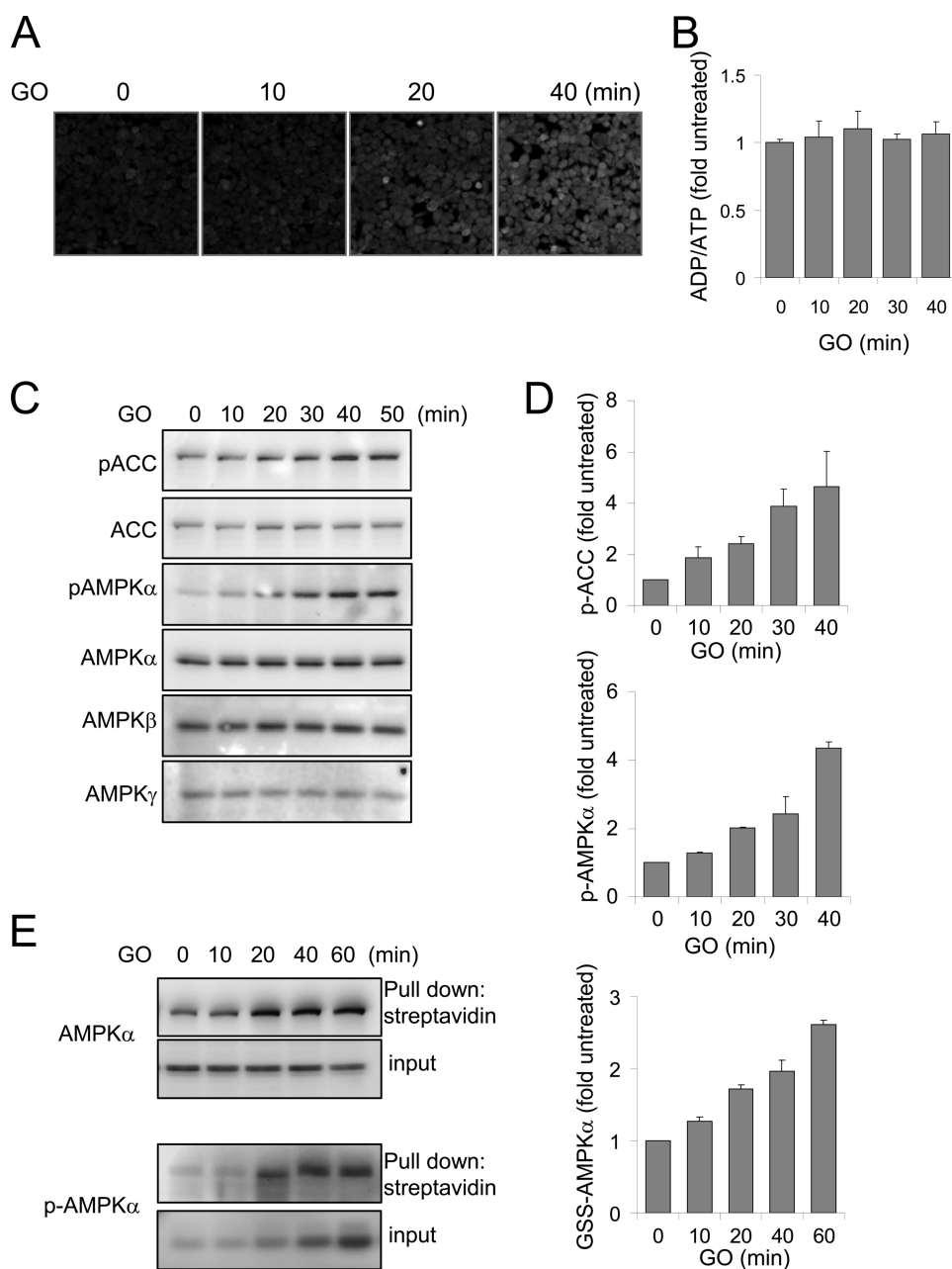


FIGURE 3. H₂O₂ induces oxidation and activation of AMPK without depletion of cellular ATP. *A–D*, HEK 293 cells loaded with DCFH-DA were cultured with GO (10 milliunits/ml) for 0, 10, 20, or 40 min, and then images were acquired using confocal microscopy (*A*). *B* shows the ADP/ATP ratios in cells incubated with GO (10 milliunits/ml) for the indicated time periods, whereas *C* and *D* show representative Western blots of AMPK subunits and phospho-ACC (pACC) and mean \pm S.D. (*error bars*) obtained from two experiments that utilized HEK 293 cells treated with GO for 0–50 min. *E*, HEK 293 cells were loaded with EE-GSH-biotin and then GO (10 milliunits/ml) included in culture medium for the indicated time period. Cell extracts were subjected to pull-down with streptavidin-agarose, followed by Western blotting with antibodies specific for AMPK α or phospho-Thr¹⁷²-AMPK (p-AMPK) (*input*, levels of AMPK or phospho-Thr¹⁷² in cell extract prior to pull-down assay; *Pull down*, the amount of AMPK or phospho-Thr¹⁷²-AMPK obtained after precipitation with streptavidin-agarose). Shown is the mean \pm S.D. obtained from two experiments.

well as oxidative modification of AMPK, those studies do not show that oxidative modification precedes activation of AMPK or is responsible for kinase activation. An alternate explanation is that activation of AMPK could have been produced by decreases in cellular levels of ATP and alteration in the ATP/AMP ratio as a result of incubation of HEK 293 cells with H₂O₂, with oxidative modification of AMPK occurring as a subsequent and independent event. Indeed, we found that exposure

of HEK 293 cells to H₂O₂ (250 μ M) for 30 min resulted in about a 30–40% decrease in intracellular levels of ATP. Because of the rapidity with which AMPK became activated after incubation of HEK 293 cells with H₂O₂, we measured AMPK activation and cellular levels of ATP in HEK 293 cells exposed to glucose oxidase (GO)-generated H₂O₂, a methodology that, in contrast to direct cellular exposure to H₂O₂, results in a more gradual increase in H₂O₂ levels in the cell culture media as well as sustained elevations in intracellular steady state concentrations of H₂O₂ (26, 59). As shown in Fig. 3, inclusion of GO (10 milliunits/ml) in the cell cultures time-dependently induced DCF fluorescence and resulted in increased phosphorylation of AMPK α and ACC, as well as *S*-glutathionylation of both AMPK α and phospho-Thr¹⁷²-AMPK α . Importantly, activation and oxidation of the AMPK α subunit was present before any changes in intracellular concentrations of ADP and ATP or of the ATP/ADP ratio occurred (Fig. 3*B*). These results show that the stimulatory effects of H₂O₂ on AMPK activity are not associated with diminished cellular ATP concentrations.

Assembly of the AMPK $\alpha\beta\gamma$ complex can be potentially affected by exposure to increased concentrations of H₂O₂. However, incubation of cells with H₂O₂ did not appear to modify the composition of AMPK $\alpha\beta\gamma$ complexes ([supplemental Fig. S1A](#)).

Direct Exposure of AMPK to H₂O₂ Increases Kinase Activity—In order to determine if H₂O₂ could directly activate AMPK, we incubated recombinant AMPK $\alpha\beta\gamma$ complex with H₂O₂ and then determined kinase activity. As shown in Fig. 4, *A* and *B*, and [supplemental Fig. S2, A and B](#),

exposure of the AMPK $\alpha\beta\gamma$ complex to H₂O₂ dose-dependently increased AMPK activity, even in the presence of AMP. In additional experiments, we have found that H₂O₂-dependent activation of AMPK resulted in autophosphorylation of the AMPK α and AMPK β subunits ([supplemental Fig. S2C](#)). These results are consistent with previous studies showing that activated AMPK undergoes autophosphorylation, with the α and β subunits being affected (60).

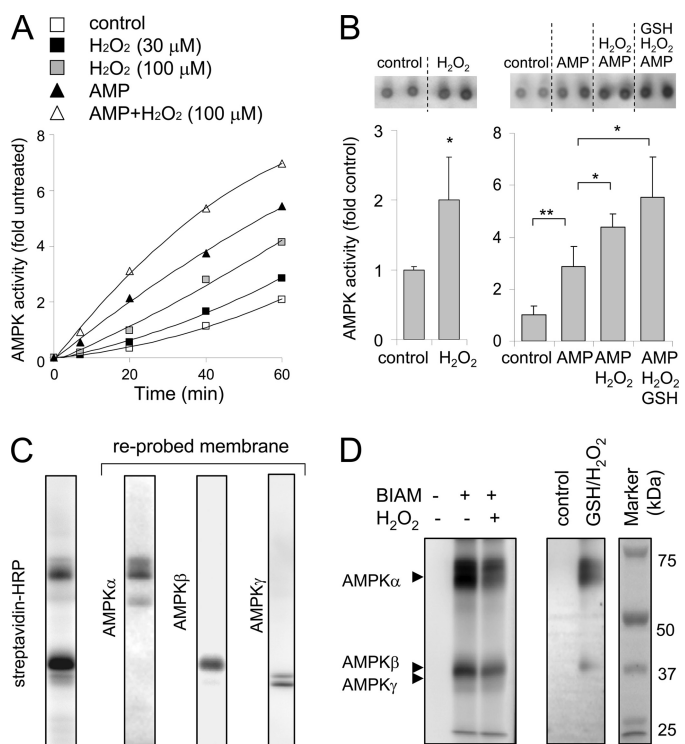


FIGURE 4. H₂O₂ directly activates AMPK. *A* and *B*, recombinant AMPK $\alpha\beta\gamma$ complex was incubated with H₂O₂ (0, 30, or 100 μ M) for 10 min, followed by inclusion of AMP (0 or 200 μ M) for an additional 10 min. *A* shows the time-dependent increase in phosphorylation of SAMS peptide by AMPK in the presence or absence of H₂O₂ or AMP and H₂O₂, whereas *B* shows representative autoradiograms (upper panels) and quantitative analysis of SAMS phosphorylation by AMPK (bottom panel). Shown is the mean \pm S.D. (error bars), $n = 3$; *, $p < 0.05$; **, $p < 0.01$. AMPK was incubated with or without H₂O₂ and a combination of H₂O₂, AMP, or GSH as indicated. GSH (0 or 100 μ M) was added to the cultures for 15 min after H₂O₂ or H₂O₂ and AMP exposure. *C* and *D*, in *C*, recombinant AMPK $\alpha\beta\gamma$ complex (0.3 μ g) was incubated with BIAM (10 μ M) for 10 min, followed by Western blotting with streptavidin-HRP. The membrane was then re-probed with antibodies specific for the AMPK α , AMPK β , and AMPK γ subunits. *D*, AMPK $\alpha\beta\gamma$ complex was incubated with H₂O₂ (0 or 100 μ M) for 10 min, followed by inclusion of BIAM (0 or 10 μ M) in the cultures for an additional 10 min. AMPK $\alpha\beta\gamma$ complex was also incubated with H₂O₂ (0 or 100 μ M) for 10 min, followed by GSH-biotin (0 or 200 μ M) for 10 min. Proteins were resolved using non-reducing SDS-PAGE, and the amounts of AMPK-BIAM or GSS-AMPK adduct formation were determined using Western blot analysis with streptavidin-HRP.

Consistent with results obtained from experiments that utilized cells cultured with H₂O₂ (Fig. 2), the increase in kinase activity produced by direct incubation of the AMPK $\alpha\beta\gamma$ complex with H₂O₂ was associated with oxidative modification of the α and β subunits, as shown by a decrease in BIAM adduct formation as well as increased S-glutathionylation of the AMPK α and AMPK β subunits (Fig. 4, *C* and *D*). These results indicate that H₂O₂ can directly increase AMPK activity and that such activation is accompanied by oxidative modification of cysteine residues within the AMPK α and AMPK β subunits.

Hydrogen Peroxide Can Activate AMPK α without Participation of β or γ Subunits—To determine whether H₂O₂-induced oxidation of the AMPK α subunit induces kinase activation independently of the effects of H₂O₂ on the other AMPK subunits, we transiently expressed AMPK α WT as well as AMPK with truncation of the C-terminal region (AMPK α 1–335 or AMPK α 1–312) in HEK 293 cells (Fig. 5). Deletion of the C-terminal region of the AMPK α subunit diminished complex for-

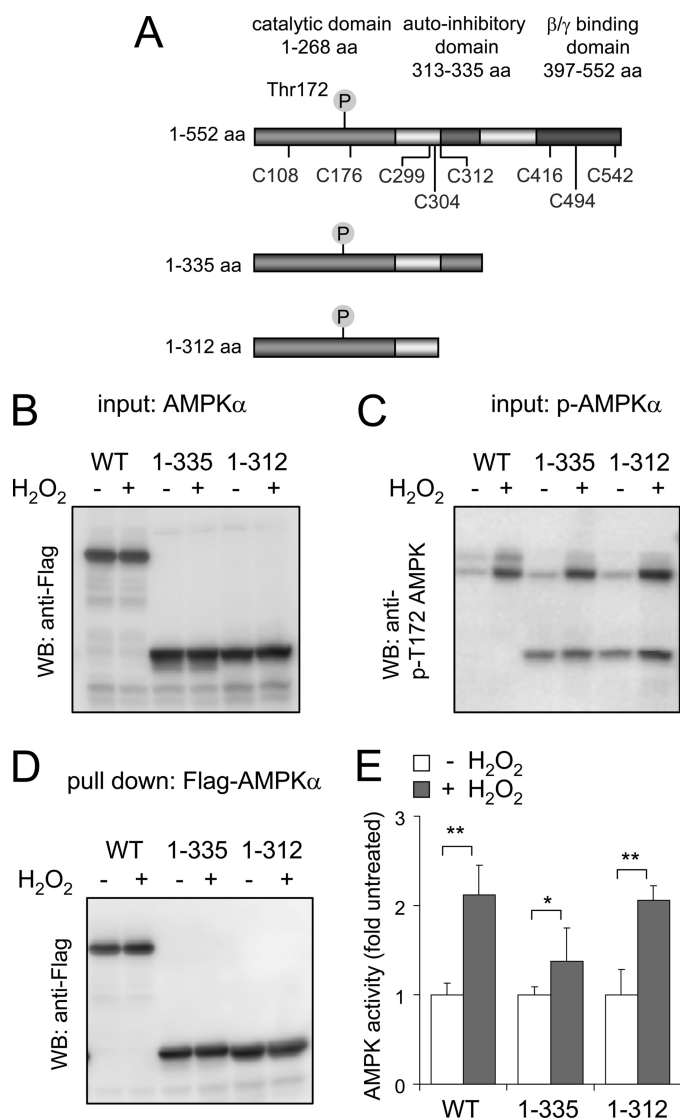


FIGURE 5. A, human AMPK. The AMPK α subunit with the location of cysteines, phosphorylation site Thr¹⁷², catalytic domain, AID, and $\beta\gamma$ binding domain and C-terminal truncation sites of AMPK α 1 as used in the experiments is shown. *B–D*, Western blots (WB) of transiently expressed FLAG-AMPK α WT, AMPK 1–335, or AMPK 1–312 obtained from HEK 293 cells treated with H₂O₂ (0 or 250 μ M) for 15 min. *B* and *C* show the level of total AMPK by probing the membrane with anti-FLAG antibodies or with antibodies to phospho-Thr¹⁷², whereas *D* shows the amount of FLAG-AMPK α or AMPK α truncation mutants obtained after pull-down with anti-FLAG-agarose. *E*, FLAG-tagged AMPK α (WT or truncation mutants) was subjected to pull-down, and then AMPK activity was determined using a radiometric assay with SAMS peptide as a substrate. Shown is the mean \pm S.D. (error bars), $n = 3$. *, $p < 0.05$; **, $p < 0.01$ compared with untreated. aa, amino acids.

mation between the α and β subunits (supplemental Fig. S1B), consistent with previous studies that have shown the importance of the β/γ binding domain located within the amino acids 397–552 region of AMPK α (7, 61). Exposure of HEK 293 cells to H₂O₂ resulted in increased activity and Thr¹⁷² phosphorylation of both wild type and truncated 1–335 or 1–312 AMPK α (Fig. 5E).

H₂O₂-dependent Oxidative Modification of Cysteine Thiols Results in Activation of AMPK α —As shown in Fig. 5, exposure of the AMPK α subunit to H₂O₂ directly increases kinase activity even after elimination of the β/γ binding domain or the AID. In particular, incubation with H₂O₂ still resulted in acti-

H₂O₂-induced AMPK Activity

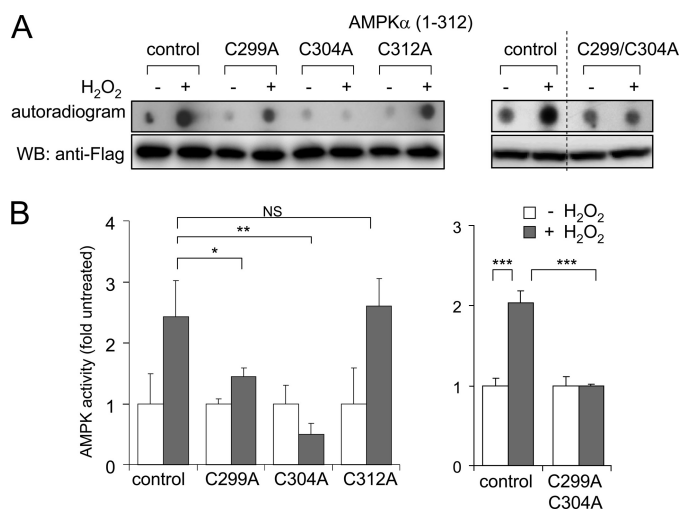


FIGURE 6. Effects of H₂O₂ on AMPK kinase activity in the AMPKα 1-312 truncation mutant and AMPKα 1-312(C299A), 1-312(C304A), 1-312(C312A), or 1-312(C299A/C304A) mutants. *A* and *B*, FLAG-tagged AMPKα WT or mutants were transiently expressed in HEK 293 cells, and the cells were treated with H₂O₂ (0 or 250 μM) for 15 min. Cell extracts were analyzed with Western blots (WB) using anti-FLAG antibodies. Kinase activity was determined using a radiometric assay with SAMS peptide as the substrate. *A*, representative autoradiograms (top) and Western blots of FLAG-AMPK (bottom). Quantitative analysis of kinase activity is shown in *B*. Shown is the mean ± S.D. (error bars), *n* = 3. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; NS, not significant.

vation of truncated AMPKα 1-335 or AMPKα 1-312. There are three cysteine residues at the C-terminal end of the AMPKα 1-312 mutant that may be oxidatively modified by exposure to H₂O₂ and contribute to the kinase activation by H₂O₂ (*i.e.* Cys²⁹⁹, Cys³⁰⁴, and Cys³¹²). To examine a potential role for these cysteines in H₂O₂-dependent regulation of AMPKα activity, point mutations of each cysteine to alanine were performed. When compared with the activation of AMPK 1-312 by exposure to H₂O₂, there was a marked decrease in the effects of H₂O₂ on kinase activity when Cys²⁹⁹ was mutated to an alanine. Mutation of Cys³⁰⁴ completely prevented activation of AMPKα 1-312 by H₂O₂. However, mutation of Cys³¹² did not result in any alteration in H₂O₂-induced kinase activation (Fig. 6). Mutation of both Cys²⁹⁹ and Cys³⁰⁴ blocked H₂O₂-induced activation of AMPKα (Fig. 6). Mutation of Cys²⁹⁹ had no effect on GSS-AMPK 1-312 adduct formation, suggesting that Cys³⁰⁴ was a specific target of H₂O₂-induced S-glutathionylation. Next, we determined if mutation of Cys²⁹⁹ and Cys³¹² affected the phosphorylation of Thr¹⁷² in AMPKα. As shown in Fig. 7C, exposure of cells to H₂O₂ produced similar levels of phosphorylation of AMPK 1-312 as compared with mutant AMPK 1-312 Cys^{299/304}. This result suggests that oxidative modification of AMPKα, subsequent to phosphorylation, is an essential step in kinase activation.

In additional experiments, we found that mutation of Cys²⁹⁹ and Cys³⁰⁴ in full-length AMPKα (amino acids 1-552) also diminished the ability of H₂O₂ but not 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, to induce kinase activity (Fig. 8).

AMPK Activity Is Increased in the Lungs of Acatalasemic Mice or Mice with Pharmacologically Induced Inhibition of Catalase—Previous studies have found that H₂O₂ at physiologically relevant concentrations can produce activation of AMPK

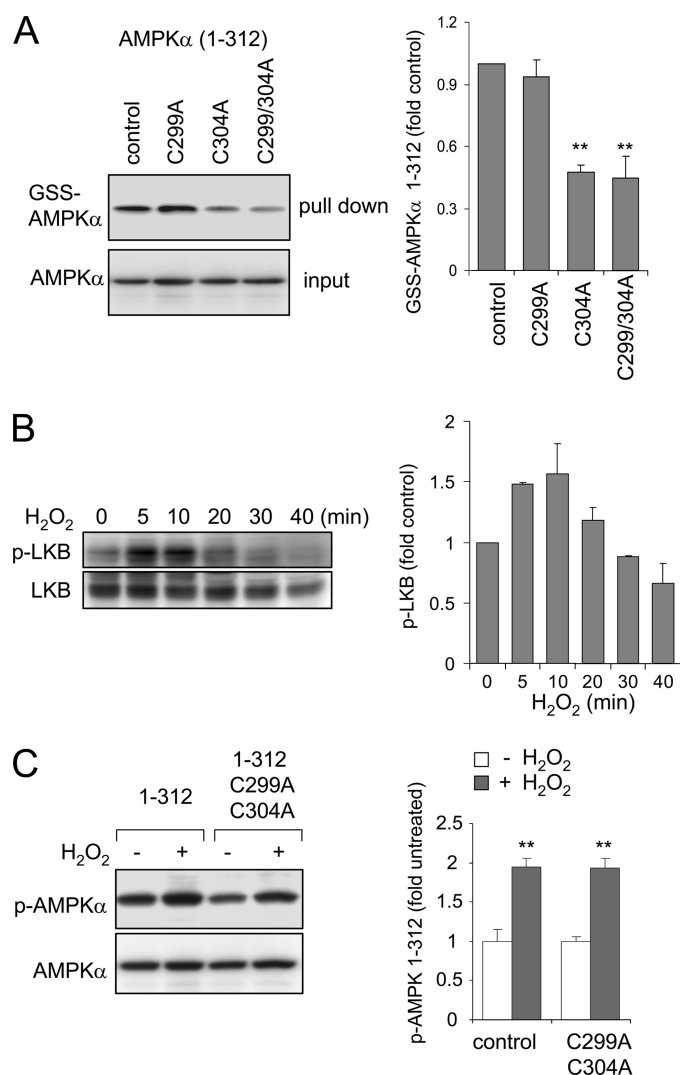


FIGURE 7. Effects of H₂O₂ on S-glutathionylation and phosphorylation of AMPKα 1-312 and AMPKα 1-312 with mutated C299A and C304A. *A*, a representative Western blot shows the amounts of GSS-AMPKα 1-312 and GSS-AMPKα 1-312 with mutated C299A and C304A obtained from HEK 293 cells loaded with biotin-GSH-EE and then treated with H₂O₂ (0 or 250 μM) for 15 min (mean ± S.D. (error bars), *n* = 3; **, *p* < 0.01). *B*, LKB1 phosphorylation (p-LKB) and total LKB1 levels were determined in HEK 293 cells treated with H₂O₂ (250 μM) for the indicated time. Representative Western blots are shown (mean ± S.D. obtained from two experiments). *C*, a representative Western blot of total and phosphorylated AMPKα 1-312 (p-AMPKα) and AMPKα 1-312 with mutated C299A and C304A obtained from HEK 293 cells treated with H₂O₂ for 15 min (mean ± S.D., *n* = 3; **, *p* < 0.01).

in vivo (33). Recently, we have shown that treatment of neutrophils with ATZ, an inhibitor of catalase, resulted in increased intracellular steady state levels of H₂O₂ as well as diminished LPS-induced proinflammatory responses, including decreased nuclear translocation of NF-κB and expression of proinflammatory cytokines (25). Given the above studies showing that direct exposure of AMPK to H₂O₂ as well as incubation of HEK 293 cells with H₂O₂ resulted in oxidation of the AMPKα subunit and enhanced kinase activity, we hypothesized that *in vivo* conditions that produce increased intracellular concentrations of H₂O₂ would also be associated with oxidative modifications of the AMPKα subunit and increased kinase activity.

As shown in Fig. 9, administration of ATZ to mice resulted in increased levels in the lungs of phosphorylated Thr¹⁷²-AMPK

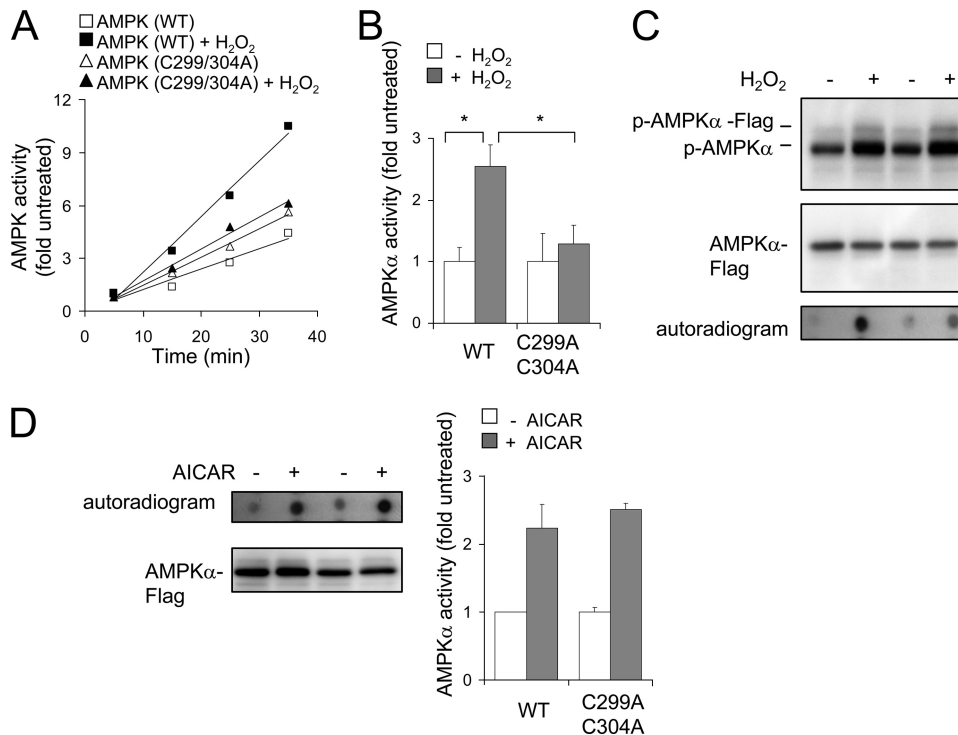


FIGURE 8. Effects of H₂O₂ on the kinase activity of wild type and mutant C299A/C304A AMPK α . A–C, AMPK α -FLAG WT or mutant AMPK α -FLAG C299A/C304A was transiently expressed in HEK 293 cells, and then the cells were treated with GO (0 or 10 milliunits/ml) for 30 min. A and B show the rate of SAMS phosphorylation by WT or mutant AMPK and mean \pm S.D. (error bars) of AMPK kinase activity ($n = 3$; *, $p < 0.05$). C, Western blots of phospho-Thr¹⁷²-AMPK α (p-AMPK α) and total AMPK α obtained from control cells and cells cultured with GO (10 milliunits/ml) for 30 min. D, a representative Western blot of AMPK α -FLAG activation and mean \pm S.D. of AMPK kinase activity obtained from cells transiently expressing WT or mutant (C299A/C304A) AMPK α and cultured with or without 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) (0 or 1 mM) for 90 min.

and AMPK activation, as shown by increased levels of the phosphorylated form of ACC (phospho-Ser⁷⁹-ACC), a downstream target of AMPK. Measurement of free cysteine thiols with BIAM labeling showed decreased amounts of BIAM-AMPK α and BIAM-AMPK β adduct formation in lung homogenates of mice given ATZ as compared with control mice treated with saline. Intratracheal LPS administration had little or no effect on AMPK phosphorylation in the lungs, whereas administration of ATZ before injection of LPS resulted in increased kinase activity. In a similar manner to acute inhibition of catalase by ATZ, increased phosphorylation and oxidation of AMPK were present in lung homogenates from acatalasemic mice (supplemental Fig. S4) compared with control mice with normal catalase function.

DISCUSSION

In this study, we demonstrated that H₂O₂ can directly activate AMPK *in vitro* and *in vivo* through a mechanism associated with enhanced oxidative modification, including S-glutathionylation of cysteine residues, of the AMPK α subunit. Previous reports had shown that exposure of various cell populations to H₂O₂ or to agents that produce increased intracellular concentrations of H₂O₂, such as by inhibiting mitochondrial electron transport, result in increased AMPK activity (29, 35, 37, 62). The ability of H₂O₂ to activate AMPK has been hypothesized to be indirect and to occur through decreasing intracellular levels of ATP and increasing the ratio of AMP to ATP, thereby

enhancing binding of AMP to the AMPK γ subunit with resultant allosteric activation of the AMPK α kinase domain (34). A recent study that utilized an AMP-insensitive mutant of the AMPK γ subunit suggested that the stimulatory effects of H₂O₂ on AMPK activation are mediated by a diminished ATP/ADP ratio (63). However, in the present experiments, we found that exposure of cells to increased levels of H₂O₂ activated and oxidized AMPK before any decrease in ATP levels or in the ATP/ADP ratio occurred. Moreover, we confirmed that H₂O₂ could directly activate AMPK by demonstrating that incubation of the AMPK $\alpha\beta\gamma$ complex with H₂O₂ increased kinase activity. However, because exposure of HEK 293 and other cell populations to H₂O₂ also results in diminished intracellular ATP levels and increased AMP/ATP ratios, in addition to increasing intracellular concentrations of H₂O₂, the ability of H₂O₂ to directly activate AMPK does not necessarily imply that this is the major mechanism by which increased generation of H₂O₂ pro-

duces activation of AMPK *in vivo*. Future experiments will be necessary to delineate the relative importance of direct oxidation of the AMPK α subunit as compared with alterations in AMP/ATP ratios in activating AMPK during pathophysiologic conditions, such as ischemia-reperfusion injury, that are associated with increased production of H₂O₂.

Although exposure to H₂O₂ resulted in oxidative modification and S-glutathionylation of both the α and β subunits of AMPK, H₂O₂-induced modification of the AMPK α subunit alone was sufficient to increase kinase activity. Of note, exposure of truncated AMPK α (amino acids 1–335), which lacks the binding domain for interaction with the AMPK β and AMPK γ subunits, to H₂O₂ still increased AMPK activity, indicating that association with the β and γ subunits was not necessary for AMPK activation by H₂O₂. Recent studies have shown that interaction between the AID and α -helix C region of the AMPK α subunit is responsible for retention of the inactive “open” conformation of the AMPK α subunit within the AMPK $\alpha\beta\gamma$ complex (7–9). However, exposure of AMPK α or AMPK α truncation mutants lacking the AID to H₂O₂ resulted in enhanced AMPK kinase activity, showing that the AID is unlikely to play an important role in this effect. Mutation of cysteine 299 decreased and mutation of cysteine 304 totally blocked the activation of AMPK α by H₂O₂, suggesting that oxidative modification of these two cysteines plays an important role in the ability of H₂O₂ to induce activation of AMPK.

H₂O₂-induced AMPK Activity

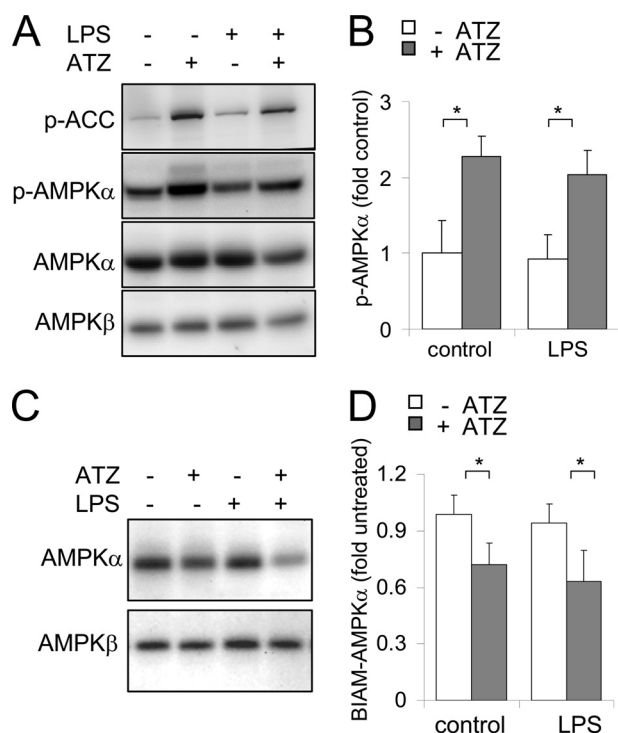


FIGURE 9. Mice with pharmacologic inhibition of catalase activity or acatalasemic mice have increased activation and oxidation of AMPK α subunit in the lungs. *A* and *B*, mice were given 50 μ l of saline or LPS (1 mg/kg) in 50 μ l of saline intratracheally and were treated with ATZ i.p. at 500 mg/kg. ATZ was administered 4 h before intratracheal saline or LPS administration. Lungs were harvested 24 h after treatment of the mice with LPS, and the levels of the AMPK α or AMPK β subunits, phospho-Thr¹⁷²-AMPK α , ACC, and phospho-Ser⁷⁹-ACC (*p*-ACC), were determined using Western blot analysis. Representative Western blots are shown in *A*, whereas quantitative analysis of AMPK phosphorylation in lung homogenates is shown in *B* (mean \pm S.D. values were obtained from 3 mice/group; *, *p* < 0.05). *C*, lung homogenates obtained from control saline-treated mice or mice given LPS, ATZ, or ATZ and LPS were incubated with BIAM, and levels of AMPK-BIAM adduct formation were determined by Western blot analysis with streptavidin-HRP. The mean \pm S.D. is shown using results from two experiments.

Although our results show that direct exposure to H₂O₂ enhances the kinase activity of AMPK, enhanced phosphorylation of AMPK was also present under such conditions. In particular, incubation of the AMPK $\alpha\beta\gamma$ complex with H₂O₂ dose-dependently increased phosphorylation of the AMPK α and AMPK β subunits. Such findings are consistent with previous studies that showed that activated AMPK undergoes autophosphorylation during activation (60).

Although H₂O₂ is a relatively weak oxidant, extracellularly generated H₂O₂ is capable of rapidly crossing cellular membranes to oxidize redox-sensitive cysteines of intracellular proteins and to modulate their activity in signaling pathways (38). The results of the present experiments, and particularly of those showing that direct exposure of the AMPK $\alpha\beta\gamma$ complex or of the AMPK α subunit to H₂O₂ increased kinase activity and diminished BIAM adduct formation, suggest that the mechanism by which H₂O₂ induces such effects is through oxidative modification of vulnerable cysteine residues. The ability of H₂O₂ to produce *S*-glutathionylation of the AMPK α and AMPK β subunits is consistent with this hypothesis. Although the present findings suggest that exposure to H₂O₂ alone is sufficient to oxidatively modify and activate AMPK, it is possi-

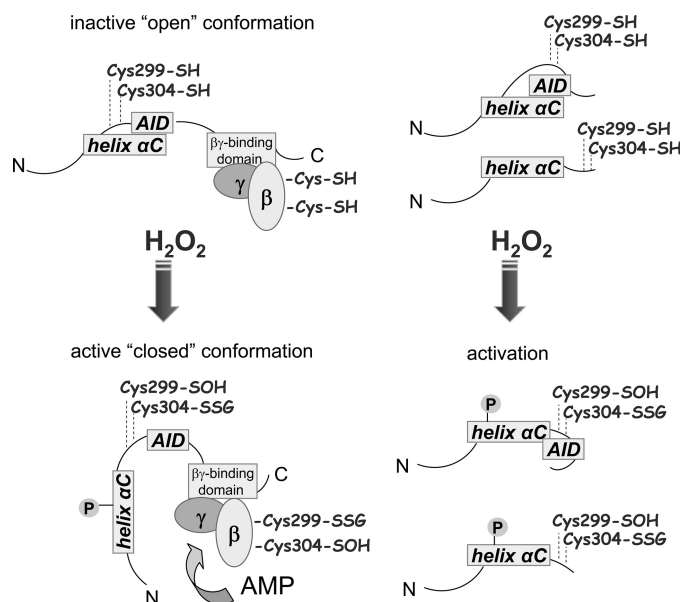


FIGURE 10. Putative mechanism of AMPK activation by H₂O₂. The AMPK $\alpha\beta\gamma$ complex is in an "open" inactive state, whereas H₂O₂ induces allosteric rearrangement to the active "closed" conformation as a result of H₂O₂-dependent oxidative modification of cysteine residues (-SOH), including *S*-glutathionylation (-SSG). Such oxidative modification, followed by dissociation of AID from α -helix C and activation of AMPK α , can be achieved without binding of β/γ subunit. In the heterotrimeric AMPK complex, oxidative modification of the α and β subunits can also facilitate AMP-dependent activation of the kinase domain.

ble that other ROS, such as hydroxyl radical, derived from H₂O₂ contribute to these effects. However, we found that metal-dependent generation of OH \cdot *in vitro* diminished the activity of AMPK (supplemental Fig. S3). Such results suggest that H₂O₂ itself, rather than derived strong oxidants, is responsible for activation of AMPK under pathophysiologic *in vivo* conditions associated with increased generation of ROS.

Similar to the effects of H₂O₂ in cell cultures, we found that increased intracellular concentrations of H₂O₂ in the lungs under *in vivo* conditions also were associated with AMPK activation (64). A role for H₂O₂ in modulating AMPK activity *in vivo* was previously reported after cardiac ischemia, when increased levels of H₂O₂ in the heart were accompanied by activation of AMPK and protection from a second ischemic event (1, 65–68). Consistent with our experiments with purified AMPK and with HEK 293 cells, we found activation of AMPK and oxidative modification of the AMPK α subunit in the lungs of acatalasemic mice and in mice treated with the catalase inhibitor ATZ, conditions in which intracellular concentrations of H₂O₂ are elevated (25).

Our experiments demonstrate a novel mechanism for AMPK activation that involves oxidative modification of the AMPK α subunit as a result of direct exposure to H₂O₂ (Fig. 10). Previous studies have shown that activation of AMPK as well as increased intracellular concentrations of H₂O₂ have potent anti-inflammatory properties, including diminished severity of LPS-induced acute lung injury (25). The present experiments suggest that at least one mechanism by which H₂O₂ exerts its anti-inflammatory effects *in vivo* is through directly activating AMPK. Future studies will be necessary to determine if the primary mechanism for the anti-inflammatory effects of

increased intracellular concentrations of H₂O₂ is through activation of AMPK.

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REFERENCES

- Baron, S. J., Li, J., Russell, R. R., 3rd, Neumann, D., Miller, E. J., Tuerk, R., Wallimann, T., Hurley, R. L., Witters, L. A., and Young, L. H. (2005) *Circ. Res.* **96**, 337–345
- Hardie, D. G., Hawley, S. A., and Scott, J. W. (2006) *J. Physiol.* **574**, 7–15
- Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (2004) *J. Clin. Invest.* **113**, 274–284
- Towler, M. C., and Hardie, D. G. (2007) *Circ. Res.* **100**, 328–341
- Stein, S. C., Woods, A., Jones, N. A., Davison, M. D., and Carling, D. (2000) *Biochem. J.* **345**, 437–443
- Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D., and Hardie, D. G. (1996) *J. Biol. Chem.* **271**, 27879–27887
- Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E., and Witters, L. A. (1998) *J. Biol. Chem.* **273**, 35347–35354
- Pang, T., Xiong, B., Li, J. Y., Qiu, B. Y., Jin, G. Z., Shen, J. K., and Li, J. (2007) *J. Biol. Chem.* **282**, 495–506
- Chen, L., Jiao, Z. H., Zheng, L. S., Zhang, Y. Y., Xie, S. T., Wang, Z. X., and Wu, J. W. (2009) *Nature* **459**, 1146–1149
- Evans, A. M., Mustard, K. J., Wyatt, C. N., Peers, C., Dipp, M., Kumar, P., Kinneer, N. P., and Hardie, D. G. (2005) *J. Biol. Chem.* **280**, 41504–41511
- Yun, H., Lee, M., Kim, S. S., and Ha, J. (2005) *J. Biol. Chem.* **280**, 9963–9972
- Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M., and Holloszy, J. O. (2000) *J. Appl. Physiol.* **88**, 2219–2226
- Dolinsky, V. W., and Dyck, J. R. (2006) *Am. J. Physiol. Heart Circ. Physiol.* **291**, H2557–H2569
- Long, Y. C., and Zierath, J. R. (2006) *J. Clin. Invest.* **116**, 1776–1783
- Hardie, D. G., and Sakamoto, K. (2006) *Physiology* **21**, 48–60
- Hardie, D. G., Scott, J. W., Pan, D. A., and Hudson, E. R. (2003) *FEBS Lett.* **546**, 113–120
- Knowler, W. C., Barrett-Connor, E., Fowler, S. E., Hamman, R. F., Lachin, J. M., Walker, E. A., and Nathan, D. M. (2002) *N. Engl. J. Med.* **346**, 393–403
- Shibata, R., Sato, K., Pimentel, D. R., Takemura, Y., Kihara, S., Ohashi, K., Funahashi, T., Ouchi, N., and Walsh, K. (2005) *Nat. Med.* **11**, 1096–1103
- Shin, E. J., Schram, K., Zheng, X. L., and Sweeney, G. (2009) *J. Cell. Physiol.* **221**, 490–497
- Gundewar, S., Calvert, J. W., Jha, S., Toedt-Pingel, I., Ji, S. Y., Nunez, D., Ramachandran, A., Anaya-Cisneros, M., Tian, R., and Lefer, D. J. (2009) *Circ. Res.* **104**, 403–411
- Lin, H. Z., Yang, S. Q., Chuckaree, C., Kuhajda, F., Ronnet, G., and Diehl, A. M. (2000) *Nat. Med.* **6**, 998–1003
- Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Zoli, M., and Melchionda, N. (2001) *Lancet* **358**, 893–894
- Zhao, X., Zmijewski, J. W., Lorne, E., Liu, G., Park, Y. J., Tsuruta, Y., and Abraham, E. (2008) *Am. J. Physiol. Lung Cell Mol. Physiol.* **295**, L497–L504
- Zmijewski, J. W., Lorne, E., Zhao, X., Tsuruta, Y., Sha, Y., Liu, G., Siegal, G. P., and Abraham, E. (2008) *Am. J. Respir. Crit. Care Med.* **178**, 168–179
- Zmijewski, J. W., Lorne, E., Zhao, X., Tsuruta, Y., Sha, Y., Liu, G., and Abraham, E. (2009) *Am. J. Respir. Crit. Care Med.* **179**, 694–704
- Zmijewski, J. W., Zhao, X., Xu, Z., and Abraham, E. (2007) *Am. J. Physiol. Cell Physiol.* **293**, C255–C266
- Strassheim, D., Asehounne, K., Park, J. S., Kim, J. Y., He, Q., Richter, D., Mitra, S., Arcaroli, J., Kuhn, K., and Abraham, E. (2004) *Am. J. Physiol. Cell Physiol.* **286**, C683–C692
- Horie, T., Ono, K., Nagao, K., Nishi, H., Kinoshita, M., Kawamura, T., Wada, H., Shimatsu, A., Kita, T., and Hasegawa, K. (2008) *J. Cell. Physiol.* **215**, 733–742
- Irrcher, I., Ljubicic, V., and Hood, D. A. (2009) *Am. J. Physiol. Cell Physiol.* **296**, C116–C123
- Sandström, M. E., Zhang, S. J., Bruton, J., Silva, J. P., Reid, M. B., Westerblad, H., and Katz, A. (2006) *J. Physiol.* **575**, 251–262
- Schulz, E., Doppeide, J., Schuhmacher, S., Thomas, S. R., Chen, K., Daiber, A., Wenzel, P., Münzel, T., and Keaney, J. F., Jr. (2008) *Circulation* **118**, 1347–1357
- Colombo, S. L., and Moncada, S. (2009) *Biochem. J.* **421**, 163–169
- Lamberts, R. R., Onderwater, G., Hamdani, N., Vredon, M. J., Steenhuisen, J., Eringa, E. C., Loer, S. A., Stienen, G. J., and Bouwman, R. A. (2009) *Circulation* **120**, S10–S15
- Choi, S. L., Kim, S. J., Lee, K. T., Kim, J., Mu, J., Birnbaum, M. J., Soo Kim, S., and Ha, J. (2001) *Biochem. Biophys. Res. Commun.* **287**, 92–97
- Emerling, B. M., Weinberg, F., Snyder, C., Burgess, Z., Mutlu, G. M., Violet, B., Budinger, G. R., and Chandel, N. S. (2009) *Free Radic. Biol. Med.* **46**, 1386–1391
- Toyoda, T., Hayashi, T., Miyamoto, L., Yonemitsu, S., Nakano, M., Tanaka, S., Ebihara, K., Masuzaki, H., Hosoda, K., Inoue, G., Otaka, A., Sato, K., Fushiki, T., and Nakao, K. (2004) *Am. J. Physiol. Endocrinol. Metab.* **287**, E166–E173
- Zhang, M., Dong, Y., Xu, J., Xie, Z., Wu, Y., Song, P., Guzman, M., Wu, J., and Zou, M. H. (2008) *Circ. Res.* **102**, 328–337
- Dröge, W. (2002) *Physiol. Rev.* **82**, 47–95
- Giustarini, D., Rossi, R., Milzani, A., Colombo, R., and Dalle-Donne, I. (2004) *J. Cell Mol. Med.* **8**, 201–212
- Dalle-Donne, I., Rossi, R., Giustarini, D., Colombo, R., and Milzani, A. (2007) *Free Radic. Biol. Med.* **43**, 883–898
- Foster, W. M., Walters, D. M., Longphre, M., Macri, K., and Miller, L. M. (2001) *J. Appl. Physiol.* **90**, 1111–1117
- Tsuruta, Y., Park, Y. J., Siegal, G. P., Liu, G., and Abraham, E. (2007) *J. Immunol.* **179**, 7079–7086
- Brass, D. M., Hollingsworth, J. W., McElvania-Tekippe, E., Garantziotis, S., Hossain, I., and Schwartz, D. A. (2007) *Am. J. Physiol. Lung Cell Mol. Physiol.* **293**, L77–L83
- Davies, S. P., Carling, D., and Hardie, D. G. (1989) *Eur. J. Biochem.* **186**, 123–128
- Zmijewski, J. W., Lorne, E., Banerjee, S., and Abraham, E. (2009) *Am. J. Physiol. Lung Cell Mol. Physiol.* **296**, L624–L634
- Wrona, M., Patel, K., and Wardman, P. (2005) *Free Radic. Biol. Med.* **38**, 262–270
- Watanabe, N., Zmijewski, J. W., Takabe, W., Umezu-Goto, M., Le Goffe, C., Sekine, A., Landar, A., Watanabe, A., Aoki, J., Arai, H., Kodama, T., Murphy, M. P., Kalyanaram, R., Darley-Usmar, V. M., and Noguchi, N. (2006) *Am. J. Pathol.* **168**, 1737–1748
- Zmijewski, J. W., Moellering, D. R., Le Goffe, C., Landar, A., Ramachandran, A., and Darley-Usmar, V. M. (2005) *Am. J. Physiol. Heart Circ. Physiol.* **289**, H852–H861
- Kim, J. R., Yoon, H. W., Kwon, K. S., Lee, S. R., and Rhee, S. G. (2000) *Anal. Biochem.* **283**, 214–221
- Choi, K. S., Park, S. Y., Baek, S. H., Dey-Rao, R., Park, Y. M., Zhang, H., Ip, C., Park, E. M., Kim, Y. H., and Park, J. H. (2006) *Prep Biochem. Biotechnol.* **36**, 65–79
- Cross, J. V., and Templeton, D. J. (2006) *Antioxid. Redox Signal.* **8**, 1819–1827
- Ying, J., Clavreul, N., Sethuraman, M., Adachi, T., and Cohen, R. A. (2007) *Free Radic. Biol. Med.* **43**, 1099–1108
- Banerjee, S., Zmijewski, J. W., Lorne, E., Liu, G., Sha, Y., and Abraham, E. (2010) *J. Biol. Chem.* **285**, 2665–2675
- Zmijewski, J. W., Banerjee, S., and Abraham, E. (2009) *J. Biol. Chem.* **284**, 22213–22221
- Kreda, S. M., Okada, S. F., van Heusden, C. A., O'Neal, W., Gabriel, S., Abdullah, L., Davis, C. W., Boucher, R. C., and Lazarowski, E. R. (2007) *J. Physiol.* **584**, 245–259
- Lazarowski, E. R., Tarran, R., Grubb, B. R., van Heusden, C. A., Okada, S., and Boucher, R. C. (2004) *J. Biol. Chem.* **279**, 36855–36864
- Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A., and Shelton, M. D. (2008) *Antioxid. Redox Signal.* **10**, 1941–1988
- Shelton, M. D., Chock, P. B., and Mieyal, J. J. (2005) *Antioxid. Redox Signal.* **7**, 348–366
- Mueller, S., Weber, A., Fritz, R., Mütze, S., Rost, D., Walczak, H., Völkl, A.,

H₂O₂-induced AMPK Activity

- and Stremmel, W. (2002) *Biochem. J.* **363**, 483–491
60. Mitchelhill, K. I., Michell, B. J., House, C. M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L. A., and Kemp, B. E. (1997) *J. Biol. Chem.* **272**, 24475–24479
61. Iseli, T. J., Walter, M., van Denderen, B. J., Katsis, F., Witters, L. A., Kemp, B. E., Michell, B. J., and Stapleton, D. (2005) *J. Biol. Chem.* **280**, 13395–13400
62. Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., and Ristow, M. (2007) *Cell Metab.* **6**, 280–293
63. Hawley, S. A., Ross, F. A., Chevtzoff, C., Green, K. A., Evans, A., Fogarty, S., Towler, M. C., Brown, L. J., Ogunbayo, O. A., Evans, A. M., and Hardie, D. G. (2010) *Cell Metab.* **11**, 554–565
64. Young, L. H. (2008) *Circulation* **117**, 832–840
65. Kudo, N., Barr, A. J., Barr, R. L., Desai, S., and Lopaschuk, G. D. (1995) *J. Biol. Chem.* **270**, 17513–17520
66. Murphy, E., and Steenbergen, C. (2008) *Physiol. Rev.* **88**, 581–609
67. Russell, R. R., 3rd, Li, J., Coven, D. L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F. J., Mu, J., Birnbaum, M. J., and Young, L. H. (2004) *J. Clin. Invest.* **114**, 495–503
68. Sukhodub, A., Jovanović, S., Du, Q., Budas, G., Clelland, A. K., Shen, M., Sakamoto, K., Tian, R., and Jovanović, A. (2007) *J. Cell. Physiol.* **210**, 224–236