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# The Regulation of AMP-Activated Protein Kinase by $H_2O_2$

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**AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine kinase, is activated by conditions leading to an increase of the intracellular AMP:ATP ratio. However, how AMPK is regulated under the oxidative stress is completely unknown. In the present study, we examined effects of the oxidative agent $H_2O_2$ on AMPK. AMPK was transiently and concentration-dependently activated by $H_2O_2$ in NIH-3T3 cells. This activation was tightly associated with an increased AMP:ATP ratio, an electrophoretic mobility shift of AMPK $\alpha$1 catalytic subunit, and an increased phosphorylation level of AMPK $\alpha$1 threonine 172, which is a major *in vitro* phosphorylation site by the upstream AMPK kinase. All of these events were significantly blocked by the pretreatment of 0.5% dimethyl sulfoxide, a potent hydroxyl radical scavenger, indicating that AMPK cascades are highly sensitive to the oxidative stress. Interestingly, a specific tyrosine kinase inhibitor, genistein, further stimulated the $H_2O_2$-induced AMPK activity by 70% without altering the AMP:ATP. Taken together, our results suggest that AMP:ATP ratio is the major parameter to which AMPK responds under the oxidative stress, but AMPK may be regulated in part by a tyrosine kinase-dependent pathway, which is independent of the cellular adenosine nucleotides level.** 

*Key Words:* **AMP-activated protein kinase; oxidative stress; $H_2O_2$; ATP; AMP; tyrosine kinase.**

Mammalian AMP-activated protein kinase (AMPK), a heterotrimeric serine/threonine kinase consisting of a catalytic $\alpha$ subunit and two regulatory $\beta$ and $\gamma$ subunits, plays a key role in the regulation of energy homeostasis (reviewed in Refs. 1–3). Elevation of intracellular AMP activates AMPK, and this is mediated via several distinct mechanisms. First, AMP causes direct allosteric activation of AMPK (4). Second, binding of AMP to AMPK renders it a better substrate for the upstream AMPK kinase (AMPKK) and a worse substrate for protein phosphatases (4, 5). Third, the upstream kinase AMPKK, which is also allosterically activated by AMP, activates AMPK by phosphorylation (4). Since some of these effects of AMP are antagonized by high concentration of ATP *in vitro* (6), it has been speculated that AMPK is sensitively regulated by the cellular AMP:ATP ratio. Indeed, AMPK is activated by stresses that deplete ATP; in mammalian cells, depletion of ATP always results in a greater increase of AMP level due to adenylate kinase, which maintains the reaction 2 ADP$\alpha$ ATP + AMP close to equilibrium (reviewed in 1). Once activated under ATP-depleting conditions, AMPK limits further ATP utilization by inhibiting key enzymes involved in ATP-consuming anabolic pathways such as fatty acid synthesis and cholesterol synthesis (7, 8). In addition, AMPK stimulates the ATP-generating pathways such as fatty acid oxidation (9, 10), glycolysis (11), and glucose uptake (12, 13). Hence, it has been proposed that AMPK play a key role in protecting the cell against ATP depletion (1–3).

The pathological or physiological conditions that activate AMPK include heat shock (14), hypoxia/ischemia in heart muscle (15), exercise in skeletal muscle (16), and metabolic toxicity (17). However, effects of the oxidative stress on AMPK cascades are completely unknown. Many studies have suggested that excess reactive oxygen species (ROS) can cause oxidative damages to macromolecules of host cell and thus play important roles in the etiology of many disease processes including cancer, atherosclerosis, ischemia/reperfusion-induced cardiac abnormalities, diabetes, and general process of aging (18). In addition to their roles in pathological processes, increasing evidence shows that ROS can be generated in a variety of cells in response to cytokines (19), growth factors (20), and agonists of receptors with seven transmembrane spans

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such as angiotensin II (21). Furthermore, it has been suggested that ROS may act as second messengers in intracellular signal transduction pathways (22, 23). Because the effects of $H_2O_2$ are thought to be mediated by the oxidative stress and ROS, herein we have investigated the regulatory mechanisms for AMPK under the oxidative stress using $H_2O_2$.

## MATERIALS AND METHODS

*Materials.* Dulbecco's modified Eagle medium (DMEM), and the other cell culture products were purchased from Life Technologies, Inc. Genistein and PD98059 were obtained from Calbiochem. Suramin and $H_2O_2$ were from Sigma. [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) was purchased from Perkin–Elmer Life Sciences.

*Cell culture and treatment.* NIH-3T3 cells were maintained in DMEM medium containing 10% calf serum. Serum starvation was achieved by incubating almost confluent NIH-3T3 cells for 3 h with Krebs–Hepes buffer (25 mM Na Hepes, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.07% BSA, and 5.5 mM glucose). Then, cells were incubated with the Krebs–Hepes buffer containing $H_2O_2$ for the indicated time. Suramin (300 $\mu$M), genistein (100 $\mu$M), or PD98059 (50 $\mu$M) were added to the Krebs–Hepes buffer for 30 min before addition of $H_2O_2$.

*Protein extracts and AMPK activity assay.* Cells were lysed with digitonin buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaF, 30 mM glycerol phosphate, 0.25% sucrose, 1 mM sodium metavanadate, 1.5 mM phenylmethylsulfonyl fluoride and 0.4 mg/ml digitonin) on ice for 2 min. AMPK was partially purified from cell lysates as recently described (24). Briefly, polyethylene glycol (PEG) 6000 was added to cell lysates to make final concentration 2.5% (w/v). After centrifugation, 2.5% PEG supernatant was collected and PEG was added to make final concentration 5% (w/v). After centrifugation, the resultant pellet was subjected to AMPK activity assay, which was determined as previously described (25) in kinase assay buffer (62.5 mM Na Hepes, pH 7.0, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, and 1 mM DTT) containing 200 $\mu$M AMP, ATP mixture (200 $\mu$M ATP and 1.5 $\mu$Ci [$\gamma$-$^{32}$P]ATP), with or without 250 $\mu$M SAMS peptide (HMR-SAMSGLHLVKRR) at 30° for 10 min. Reaction was terminated by spotting the reaction mixture on phosphocellulose paper (P81), and the paper was extensively washed with 150 mM phosphoric acid. The radioactivity was measured by scintillation counter.

*Nucleotide analysis.* The intracellular level of ATP and AMP was measured as previously described with following modification (14). NIH-3T3 cell suspension (1 ml) in PBS was mixed with 100 $\mu$l of 55% (w/v) $HClO_4$, and protein precipitated by perchloric acid was removed by centrifugation. The supernatant fraction was extracted twice with 10% excess by volume of 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. Nucleotides were then separated on a Kromasil KR100-$NH_2$ column (0.46 × 25 cm) at a flow rate of 1.5 ml min$^{-1}$. The mobile phase consisted of ammonium phosphate buffer and acetonitrile, and the following gradient conditions were used. The column was equilibrated for 10 min with a mixture of 50 mM ammonium phosphate, pH 4.0, and acetonitrile (60:40, v/v). After injection of the sample, the analytical condition was maintained isocratically at that ratio for 20 min. The percentage of acetonitrile was then raised within 5 min to 100% (v/v) using a linear gradient. Separation was performed on a Waters HPLC system with a Model 486 ultraviolet detector set at 260 nm. Areas of peaks were calculated using Millenium software, and converted to molar quantities by comparison with AMP and ATP standards.

*Production of anti-phosphoThr$^{172}$ AMPK $\alpha$ antibody.* Peptide corresponding to amino acid 165-179 of AMPK $\alpha$1 (SDGEFLRpTSCGSPNY) was synthesized at Howard Hughes Medical Institute, Protein Structure Laboratory of University of California (San Francisco, CA). It was then coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL) and used to immunize rabbits from Rockland Inc. (Gilbertsville, PA). Anti-phospho Thr$^{172}$ antibody was purified using the immunogenic peptides coupled to Thio-gel columns, and it was further purified by passing through a second column coupled with a similar peptide containing an unphosphorylated Thr$^{172}$ residue.

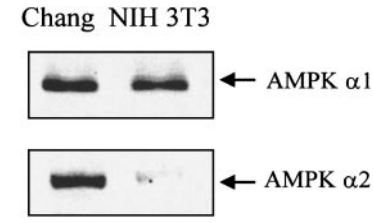


**FIG. 1.** AMPK $\alpha$1 is a predominantly expressed isoform in NIH-3T3 cell. Protein extracts (20 $\mu$g) of NIH-3T3 cell and Chang liver cell were separated by 10% SDS/polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted with AMPK $\alpha$1- or $\alpha$2-specific antibody. The arrow indicates the $\alpha$ catalytic subunit of AMPK with a molecular mass 63 kDa.

## RESULTS

### *Effects of $H_2O_2$ on AMPK Activity and Intracellular AMP:ATP Ratio in NIH-3T3 Cell*

To investigate the regulatory mechanisms for AMPK under the oxidative stress, NIH-3T3 cells have been used in the present study, and we first examined the expression level of two isoforms of AMPK catalytic subunit ($\alpha$1 and $\alpha$2) in these cells. It is known that AMPK $\alpha$1 is ubiquitously expressed in every tissue, whereas $\alpha$2 is highly expressed in liver, muscle and heart (26, 27). Recent studies demonstrated that these two isoforms show different biochemical nature in respect to AMP-dependence and subcellular localization (28), suggesting that each isoform may a have tissue-specific role. Immunoblot analysis with AMPK $\alpha$1- and $\alpha$2-specific antibody shows that $\alpha$1 is a predominantly expressed form in NIH-3T3 cell, whereas two isoforms were detected in Chang liver cell (Fig. 1).

To determine effects of the oxidative stress on AMPK, we examined time- and dose-dependent effects of $H_2O_2$ on AMPK activity; $H_2O_2$ is readily converted to hydroxyl radicals via Fenton reaction, and the hydroxyl radical is considered potentially the most potent oxidant in biological systems (18). NIH-3T3 cells were serum-starved for 3 h in Krebs–Hepes buffer and then exposed to $H_2O_2$. Time course studies (Fig. 2A) revealed that $H_2O_2$ (300 $\mu$M) induced a rapid but transient activation of AMPK, about 4- to 5-fold within 5 min. Then, the AMPK activity declined but remained slightly elevated until 20 min exposure, and thereafter gradually returned to the basal level in 1 h. AMPK activation by $H_2O_2$ was concentration-dependent, and the maximum activity was observed at 300–600 $\mu$M

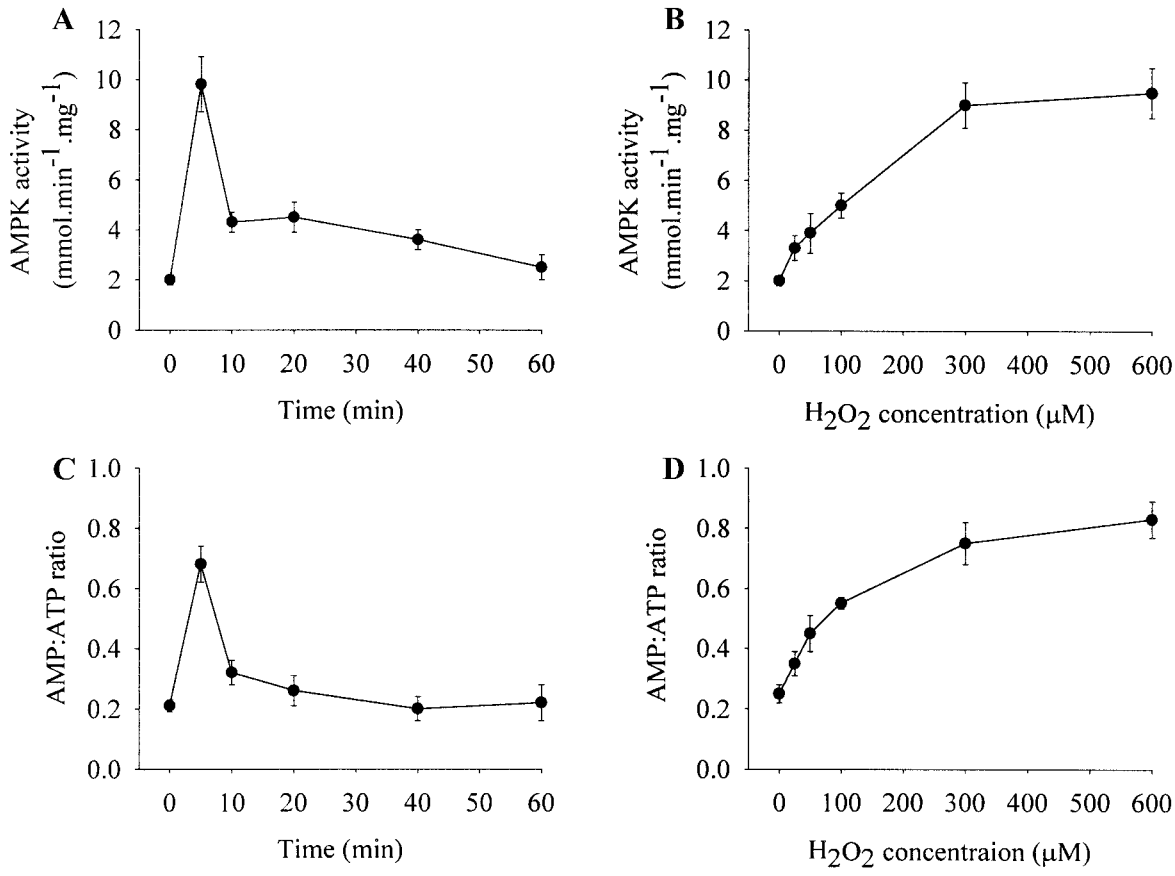


**FIG. 2.** Time- and concentration-dependent effects of $H_2O_2$ on AMPK activity and intracellular levels of ATP and AMP. NIH-3T3 cells were serum-starved for 3 h and then treated with 300 $\mu$M of $H_2O_2$ for the indicated time period (A, C) or with the indicated concentration of $H_2O_2$ for 5 min (B, D). Partially purified AMPK was subjected to the activity assay using SAMS peptide as a substrate in the presence of 200 $\mu$M AMP. The data of AMPK activity at each condition represent the mean ± SE for 5 independent experiments of duplicate (A, B). Under the identical conditions of A and B, the intracellular levels of ATP and AMP were measured and expressed as the AMP:ATP ratio (C, D). Data are mean values ± SE for two independent experiments of duplicate.

when NIH-3T3 cells were exposed to $H_2O_2$ for 5 min (Fig. 2B). To understand the regulatory mechanisms, by which AMPK is activated by $H_2O_2$, we next measured an intracellular ATP and AMP level under the identical conditions, and these are expressed as an AMP:ATP ratio (Figs. 2C and 2D). $H_2O_2$ caused a rapid depletion of cellular ATP and a simultaneous accumulation of cellular AMP. The AMP:ATP ratio peaked at 5 min following $H_2O_2$ exposure, and then immediately returned to the basal level within 20 min (Fig. 2C). This ratio also increased in a concentration-dependent manner, and the maximum effect was observed at 300–600 $\mu$M of $H_2O_2$ (Fig. 2D). Overall, AMPK activity correlates well with the intracellular AMP:ATP ratio. Pretreatment for 20 min with 0.5% dimethyl sulfoxide (DMSO), a potent hydroxyl radical scavenger (29), significantly blocked the $H_2O_2$-induced AMPK activation as well as ATP depletion (Fig. 3). Taken together, these results suggest that AMPK is highly sensitive to the oxidative stress and that the cellular AMP:ATP ratio is

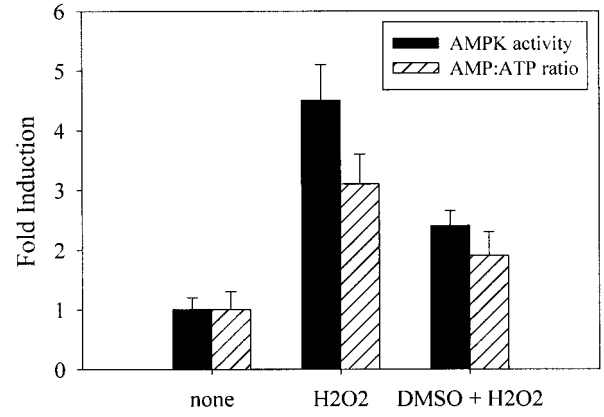


**FIG. 3.** NIH-3T3 cells were serum-starved for 3 h and then treated with 300 $\mu$M of $H_2O_2$ for 5 min. DMSO (0.5%) was added for 20 min before the stimulation of $H_2O_2$. At each condition, the AMPK activity and intracellular AMP:ATP ratio were measured, and they were expressed as a fold induction. The results are the mean ± SE for two independent assays of duplicate.

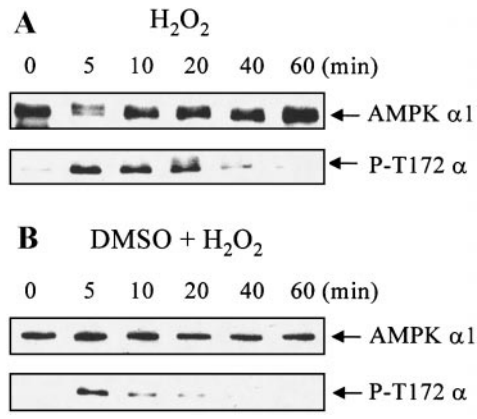


**FIG. 4.** The phosphorylation level of AMPK $\alpha$1 Thr$^{172}$ during $H_2O_2$ treatment. NIH-3T3 cells were serum-starved for 3 h and then treated with 300 $\mu$M of $H_2O_2$ for the indicated period of time (A), and DMSO (0.5%) was added for 20 min before $H_2O_2$ addition (B). The AMPK $\alpha$1 expression level (AMPK $\alpha$1) and the phosphorylation level of AMPK $\alpha$1 Thr$^{172}$ (P-T172 $\alpha$) were determined by immunoblot analysis using AMPK $\alpha$1-specific antibody and phosphospecific AMPK $\alpha$ Thr$^{172}$ antibody, respectively.

the major parameter to which AMPK responds under the condition.

*Activation of AMPK by $H_2O_2$ Was Associated with an Increase in the Phosphorylation Level of Thr$^{172}$ of AMPK $\alpha$1 Catalytic Subunit*

Any allosteric effect of AMP within the cell does not survive homogenization and so cannot be readily measured in cell extracts. Consequently, the AMPK activity of Figs. 2 and 3 should reflect the effect of the covalent modification of the enzyme. Recent studies revealed that AMPK $\alpha$1 contains multiple phosphorylation sites including autophosphorylation site (30). Among these, Thr$^{172}$ that resides in the catalytic core region was identified as the major *in vitro* phosphorylation site by AMPKK (31). Since AMPKK itself is also activated by 5′AMP *in vitro* (4), we next examined the possibility that phosphorylation of Thr$^{172}$ on AMPK $\alpha$1 is a mechanism responsible for AMPK activation under the oxidative stress. During 1 h treatment with $H_2O_2$ (300 $\mu$M), the expression level of AMPK $\alpha$1 was not affected, but two distinct bands of $\alpha$1 subunit with a different electrophoretic mobility were observed following 5 min exposure to $H_2O_2$ (Fig. 4A, upper panel). Immunoblot analysis with an antibody that specifically recognizes the phosphorylated Thr$^{172}$ of AMPK $\alpha$ shows that $H_2O_2$ markedly stimulated phosphorylation of this residue within 5 min, and then its level declined in accordance with the enzyme activity (Fig. 4A, lower panel). Pretreatment of DMSO prior to $H_2O_2$ exposure distinctively blocked the $H_2O_2$-induced mobility shift of AMPK $\alpha$1 (Fig. 4B, upper panel) as well as the $H_2O_2$-induced phosphorylation of Thr$^{172}$ of AMPK $\alpha$1 (Fig. 4B, lower panel). Therefore, these results suggest that the upstream kinase AMPKK is also activated in response to ATP depletion and that phosphorylation of Thr$^{172}$ is indeed critical for the regulation of AMPK activity in intact cells. In addition, the mobility shift of AMPK $\alpha$1 is likely to be due to the phosphorylation of Thr$^{172}$. No other post-translational modification except phosphorylation has been identified for AMPK $\alpha$1.

*Inhibition of Tyrosine Kinases potentiates the $H_2O_2$-Induced AMPK Activation without Altering the Intracellular AMP:ATP Ratio*

$H_2O_2$ not only depletes the intracellular ATP as demonstrated in this study, but also it mimics the actions of receptor-binding proteins (32–34) and activates key tyrosine kinases involved in the earliest response to various extracellular stimuli (34). It is also known that extracellular signal-regulated kinase (Erk) is activated and protects cells following $H_2O_2$ exposure (35). Next, we tested whether these signal pathways are involved in the regulation of AMPK under the oxidative stress condition. In fact, little is known about the regulatory mechanisms for AMPK except the allosteric regulation by ATP and AMP. NIH-3T3 cells were pretreated with suramin (300 $\mu$M), genistein (100 $\mu$M), or PD98059 (50 $\mu$M) for 30 min and then challenged with $H_2O_2$ (300 $\mu$M) for 5 min. Suramin blocks ligand-receptor interactions and can inhibit Erk activation induced by epidermal growth factor, short wavelength UV radiation, and $H_2O_2$ (35, 36). Genistein and PD98059 specifically inhibit tyrosine kinases and MAPK/Erk kinase, respectively. Pretreatment of genistein (100 $\mu$M) further activated the $H_2O_2$-induced AMPK activity approximately 70% without altering the AMP:ATP ratio (Fig. 5A), suggesting that AMPK can be regulated by a novel regulatory mechanism that is independent of the intracellular ATP and AMP level. This potentiation was associated with a more evident gel mobility shift of AMPK $\alpha$1 than $H_2O_2$ alone-induced one (Fig. 5B), indicating that an additional covalent modification besides phosphorylation of Thr$^{172}$ occurred under the condition. Nature of this modification is likely to be phosphorylation of unknown residue(s) because treatment of cell extracts with protein phosphatase-2C significantly diminished the genistein-induced gel mobility shift (data not shown). In contrast, suramin and PD98059 exerted practically no effect on the $H_2O_2$–induced AMPK activity, AMP:ATP ratio, and phosphorylation level of AMPK $\alpha$1 Thr$^{172}$ (Fig. 5). In conclusion, these results suggest that AMPK can be regulated in part via a tyrosine kinase-dependent pathway under the oxidative stress, which involves additional phosphorylation site(s) of AMPK $\alpha$1 other than Thr$^{172}$.

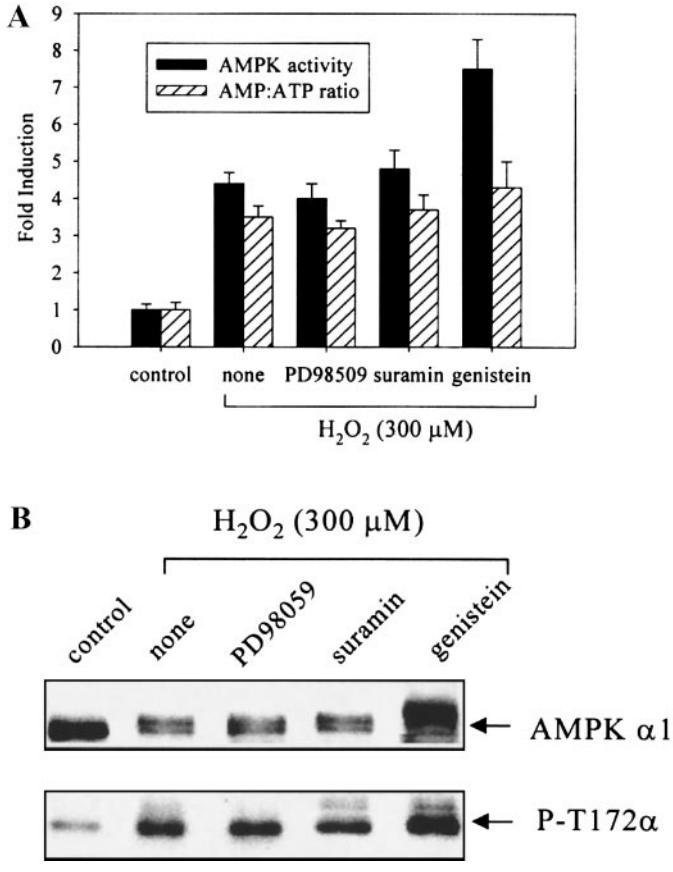


**FIG. 5.** NIH-3T3 cells were pretreated with PD98059 (50 $\mu$M), suramin (300 $\mu$M) or genistein (100 $\mu$M) for 30 min, and then challenged with $H_2O_2$ (300 $\mu$M) for 5 min. (A) At each condition, the AMPK activity and intracellular AMP:ATP ratio were measured, and they were expressed as a fold induction. The results represent the means ± SE for two independent experiments of duplicate. (B) Under the identical conditions, the AMPK $\alpha$1 expression level (AMPK $\alpha$1) and the phosphorylation level of AMPK $\alpha$1 $Thr^{172}$ (P-T172 $\alpha$) were determined by immunoblot analysis using each specific antibody.

## DISCUSSION

In the present study, we demonstrated that AMPK cascades were highly sensitive to the oxidative stress. AMPK cascades have been postulated to respond mainly to the intracellular level of AMP or AMP:ATP ratio (1–3), and a part of our result supports this speculation. Consistent with many reports showing that $H_2O_2$ evokes the intracellular ATP depletion (37–40), extracellular administration of non-lethal concentration of $H_2O_2$ (300 $\mu$M) caused a rapid depletion of ATP and accumulation of AMP in NIH-3T3 cells (Fig. 2C). Under our experimental conditions, the level of AMP transiently increased following $H_2O_2$ exposure, reaching maximum at 5 min, and then rapidly declined. The transient accumulation of AMP may be due to the rapid conversion of AMP to inosine monophosphate (IMP) because $H_2O_2$ is also known to dramatically activate AMP-deaminase (40), which converts AMP to IMP. The profile of AMPK activation by $H_2O_2$ was tightly associated with an increase of the AMP:ATP ratio (Fig. 2). Moreover, DMSO, a potent hydroxyl radical scavenger, significantly blocked AMPK activation as well as ATP depletion caused by $H_2O_2$ (Fig. 3). Therefore, these results indicate that the change in the intracellular level of ATP and AMP caused by $H_2O_2$ is a major signal for AMPK regulation under the oxidative stress. In addition, the correlation between AMPK activity and the phosphorylation level of AMPK $\alpha$1 $Thr^{172}$ (Fig. 4) indicates that phosphorylation of AMPK by AMPKK is indeed a critical step for AMPK activation in intact cell in response to ATP depletion.

On the other hand, we also suggest that AMPK can be regulated in an adenosine nucleotides-independent manner, which involves tyrosine kinases; the pretreatment of genistein, a specific inhibitor of tyrosine kinases, further stimulated the $H_2O_2$-induced AMPK activity without altering the AMP:ATP ratio (Fig. 5A). This potentiation is likely to be mediated by phosphorylation of some other residues than $Thr^{172}$ on AMPK $\alpha$1. AMPK $\alpha$1 underwent a gel mobility shift due to phosphorylation of $Thr^{172}$ in response to $H_2O_2$ stimulation (Fig. 4), and the $H_2O_2$-induced mobility was further retarded by genistein pretreatment (Fig. 5B). This additional gel mobility shift induced by genistein was significantly diminished by protein phosphatase-2C treatment (data not shown). Therefore, there seems to be some other activating phosphorylation sites on AMPK $\alpha$1 in addition to $Thr^{172}$, and this speculation is in line with the previous observation that AMPK $\alpha$1 mutant in which threonine 172 was replaced with aspartic acid was still inactivated by protein phosphatase treatment (1). Since the genistein-induced modification occurred without changes of the intracellular AMP:ATP ratio (Fig. 5), another AMPK kinase, which is distinct from one that has been defined up to date, may regulate the downstream AMPK in adenosine nucleotides-independent manner. Furthermore, this result (Fig. 5) raises a possibility that AMPK responds to extracellular signals because many tyrosine kinases including receptor and non-receptor type are involved in the earliest response to various extracellular stimuli. In fact, several reports support this possibility although intracellular signals such as ratios of AMP:ATP (1) or creatine:phosphocreatine in muscle (41) are obviously major signals for AMPK regulation. For example, insulin slightly inhibited AMPK activity in Fao cell (42), heart (43), and muscle (44). In addition, AMPK was activated by the stimulation of Gq-coupled receptor (45). Moreover, we recently demonstrated that AMPK can inhibit insulin-like growth factor-1-induced Erk activation (46). Therefore, although further studies are required, there may be a cross talk between AMPK systems and the signal transduction pathways induced by extracellular stimuli.

Reactive oxygen species (ROS) are constantly generated in all aerobic organisms as the result of the partial reduction of molecular oxygen under physiological and pathological conditions. Since ROS can non-specifically react with macromolecules and damage various cellular structures and functions, oxidative damage has been linked to a variety of human diseases and general process of aging (18). Accordingly, many efforts have been devoted to identify possible new targets of ROS in relation to these disorders. Although AMPK itself is not likely to be a direct target of ROS, our demonstration that AMPK cascades are highly sensitive to the oxidative stress implies that AMPK may play significant roles in these disorders.

## ACKNOWLEDGMENTS

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