

Enhanced Production of Adenosine Triphosphate by Pharmacological Activation of Adenosine Monophosphate-Activated Protein Kinase Ameliorates Acetaminophen-Induced Liver Injury

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The hepatic cell death induced by acetaminophen (APAP) is closely related to cellular adenosine triphosphate (ATP) depletion, which is mainly caused by mitochondrial dysfunction. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a key sensor of low energy status. AMPK regulates metabolic homeostasis by stimulating catabolic metabolism and suppressing anabolic pathways to increase cellular energy levels. We found that the decrease in active phosphorylation of AMPK in response to APAP correlates with decreased ATP levels, *in vivo*. Therefore, we hypothesized that the enhanced production of ATP via AMPK stimulation can lead to amelioration of APAP-induced liver failure. A769662, an allosteric activator of AMPK, produced a strong synergistic effect on AMPK Thr172 phosphorylation with APAP in primary hepatocytes and liver tissue. Interestingly, activation of AMPK by A769662 ameliorated the APAP-induced hepatotoxicity in C57BL/6N mice treated with APAP at a dose of 400 mg/kg intraperitoneally. However, mice treated with APAP alone developed massive centrilobular necrosis, and APAP increased their serum alanine aminotransferase and aspartate aminotransferase levels. Furthermore, A769662 administration prevented the loss of intracellular ATP without interfering with the APAP-mediated reduction of mitochondrial dysfunction. In contrast, inhibition of glycolysis by 2-deoxy-glucose eliminated the beneficial effects of A769662 on APAP-mediated liver injury. In conclusion, A769662 can effectively protect mice against APAP-induced liver injury through ATP synthesis by anaerobic glycolysis. Furthermore, stimulation of AMPK may have potential ther-

apeutic application for APAP overdose.

INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug and is also the most frequent cause of acute liver injury in many countries (Larson et al., 2005). APAP is metabolized to N-acetyl-*p*-benzoquinone imine (NAPQI) by the cytochrome P450 family of oxidase enzymes (Dahlin et al., 1984). NAPQI causes rapid depletion of cellular glutathione (GSH), which increases covalent binding in cytoplasm and mitochondria (Cohen and Khairallah, 1997; Nelson, 1990). Elevated protein adducts produce reactive oxygen species (ROS), which stimulate the activation of various mitogen-activated protein (MAP) kinases (McGill et al., 2012). Prolonged activation of c-jun-N-terminal kinase (JNK) in particular, amplifies the oxidant stress and peroxynitrite formation, which opens the membrane permeability transition (MPT) pores (Packer et al., 1997; Saito et al., 2010). Severe mitochondrial dysfunction ultimately leads to the cessation of oxphos-dependent ATP synthesis. N-acetylcysteine (NAC) is the only known antidote that prevents progression to liver failure. However, NAC needs to be administered promptly following an overdose of APAP to avoid severe morbidity and mortality (Polson and Lee, 2005). Therefore, there is an urgent need for alternative hepatoprotective agents.

Adenosine monophosphate-activated protein kinase (AMPK) is a serine-threonine kinase heterotrimer that consists of a catalytic α subunit and regulatory β , and γ subunits (Hardie and Sakamoto, 2006; Towler and Hardie, 2007). In mammals, AMPK is activated by metabolic stress such as hypoglycemia, hypoxia, and exercise that increase intracellular AMP, as well as by other allosteric effectors (Hardie and Sakamoto, 2006). AMPK stimulates ATP production by switching off anabolic processes and turning on catabolic pathways (Yang et al., 2010). In addition to regulation of energy homeostasis, AMPK has a cytoprotective role in hepatocytes exerted by inhibition of apoptosis, regulation of mitochondrial biogenesis, protection against mitochondrial injury, and activation of autophagy (Ido et al., 2002; Peralta et al., 2001). A recent study also showed that AMPK plays a critical role in APAP-induced cytotoxicity (Saberli et al., 2014).

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In this study, we explored whether pharmacological activation of AMPK can ameliorate APAP-induced liver injury and investigated its underlying mechanisms of action in rodent models.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies (Abs) against apoptosis inducing factor (AIF), phosphorylated AMPK (pAMPK), and total AMPK (tAMPK) were purchased from Cell Signaling (USA). Abs against endonuclease G (endo G) and α -tubulin were purchased from Abcam (UK). The anti-nitrotyrosine Ab was purchased from Upstate Biotechnology (USA). A769662 and Tocrisolve 100 were purchased from Tocris Bioscience (Bristol, UK). The 2-deoxy-D-glucose (2DG) was from Sigma-Aldrich (USA). All other reagents were purchased from Calbiochem (USA).

Animal experiments

All animal experiments were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (Korea). Male C67BL/6N mice were housed in a room maintained at a constant temperature (20–22°C) and kept on a 12:12 h light:dark schedule. The mice were fasted for 16 h prior to commencement of experiments. All treatments were administered intraperitoneally (ip). APAP was dissolved in warm sterile water while A769662 was dissolved in Tocrisolve 100 and administered to mice 1 h prior to APAP treatment. The 2DG was dissolved in sterile water and administered with the APAP. Following treatments, the mice were killed, the livers were removed, and the tissue immediately frozen or fixed in formalin.

Histological analysis

Paraffin-embedded liver sections (5 μ m) were used for hematoxylin and eosin (H&E) staining. Following deparaffinization and rehydration, sections were stained with hematoxylin for 1 min and eosin for 30 s, according to the manufacturer's protocol. Liver sections were stained by using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit (Millipore, USA) according to the manufacturer's procedure.

Isolation and culture of mouse primary hepatocytes

Primary hepatocytes were isolated from 8-week-old male mice. Briefly, mice were anesthetized with 2,2,2-tribromoethanol and tert-amyl alcohol (avertin, 2.5%), and their livers were perfused with liver perfusion I solution (142 mM sodium chloride, NaCl; 6.7 mM potassium chloride, KCl; 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES; 2.5 mM ethylene glycol tetraacetic acid, EGTA; pH 7.4) at 8 ml/min for 2 min, followed by perfusion II solution (66.7 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.8 mM calcium chloride; CaCl₂, pH 7.6) containing type IV collagenase (25 mg/50 ml, Sigma-Aldrich, USA) for 4 min, by using a perista pump (ATTO, Japan). Hepatocytes were harvested by using meshes and purified at 1250 rpm for 10 min with Percoll (Sigma-Aldrich, USA) containing 1X Hank's balanced salt solution (HBSS; Invitrogen, USA). The hepatocytes were counted by using the trypan blue exclusion method, and then seeded onto collagen-coated 6-well plates (5 \times 10⁵ cells/well) in M199 medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA). After 4 h, the medium was replaced with new Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) containing 10% FBS. Hepatocytes

were incubated for 24 h before the commencement of all experiments.

JC-1 assay

A JC-1 assay kit (Abcam, UK) was used to measure the depolarization of the mitochondrial membrane potential. Primary hepatocytes were seeded in 96-well plates at a density of 1 \times 10⁴ cells/ml. The following day, the cells were treated with A769662 (10 μ M) with or without APAP (10 mM). After 12 h, the cells were further incubated with JC-1 dye at 37°C in a 5% CO₂ incubator for 20 min. Aggregate formation by JC-1 was finally estimated by using the ratio of fluorescence emissions at 530 and 590 nm.

Plasma analysis

At the end of the experimental period, mice were euthanized by cervical dislocation. Blood samples were collected from the orbital venous sinus for the determination of concentrations of plasma biomarkers. Plasma was obtained by centrifugation of the blood at 10,000 \times g for 5 min at 4°C and stored at -70°C until required for analysis. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by using an automatic chemical analyzer (Hitachi 7150, Japan).

ROS measurements

Liver tissue was fractionated by first homogenizing it with a tight-fitting pestle in lysis buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 5 mM HEPES. Homogenates were then centrifuged at 600 \times g for 10 min at 4°C, and the supernatants were collected and further centrifuged at 17,000 \times g for 10 min at 4°C. The mitochondrial-enriched pellets obtained were washed twice to minimize putative contamination with cytosolic proteins. The supernatants were centrifuged again at 17,000 \times g for 10 min at 4°C, and the cytosolic protein pellets were obtained. The isolated proteins were quantified by using the Bradford assay.

The total levels of ROS produced were determined by the oxidative conversion of the nonfluorescent 2',7'-dichlorofluorescein diacetate (H₂DCFDA, Invitrogen) to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the cytosolic and mitochondrial liver fractions. Briefly, liver extracts (100 μ l) were incubated at 37°C for 60 min with 100 μ l of a solution of 200 μ M H₂DCFDA in PBS. Fluorescence was recorded at 485 nm (excitation) and 527 nm (emission) with a Victor3 1420 Multilabel Counter (Perkin Elmer, USA) and normalized to the protein content.

Western blot analysis

Liver tissue samples were homogenized in tissue lysis buffer (20 mM HEPES, pH 7.0; 2 mM EGTA, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 150 mM NaCl, and 20 mM glycerol-2-phosphate). The homogenates were centrifuged at 16,500 \times g for 15 min to remove the cell debris. The remaining separated supernatant was centrifuged again at 16,500 \times g for 15 min. The proteins were separated by electrophoresis on 10–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Primary Abs were used for the immunoblotting assay.

Glutathione (GSH) assay

The assay samples were prepared by homogenizing about 200 mg of liver tissue, in cold buffer [50 mM 2-(N-morpholino)ethanesulfonic acid, MES or phosphate; pH 7.0; 1 mM ethylenediaminetetraacetic acid, EDTA] and centrifuging at

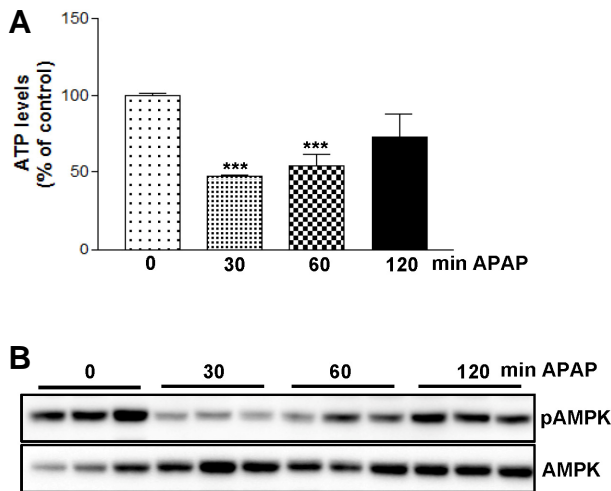


Fig. 1. Time-dependent changes in adenosine triphosphate (ATP) and adenosine monophosphate-activated protein kinase (AMPK) phosphorylation by acetaminophen (APAP). Eight-week-old C57BL/6N male mice were intraperitoneally administered 400 mg/kg APAP. Liver tissue samples were collected at indicated time points following APAP treatment. (A) ATP concentration was measured in the liver samples from each group. (B) AMPK activation was estimated by immunoblot analysis by using antibodies (Abs) against the phosphorylated (pT172) or total AMPK; $n = 5$, $*P < 0.05$, $***P < 0.001$ as compared to time zero.

10,000× *g* for 15 min at 4°C. The supernatants were deproteinated by using an equal volume of metaphosphoric acid (MPA, 1.2 M) reagent to the sample. GSH concentrations were determined by using a GSH assay Kit (BioVision Research Products, USA) according to the manufacturer's instructions.

Measurement of ATP content

For ATP measurement, liver tissue samples were lysed with ice-cold 6% perchloric acid and centrifuged at 13,000 rpm for 10 min at 4°C. The acidic extracts were neutralized with a solution containing 2 M potassium hydroxide (KOH), 2 mM EDTA, and 50 mM 3-(N-morpholino)propanesulfonic acid (MOAPS). ATP levels were estimated by using an assay kit according to the manufacturer's instructions (BioVision Research Products, USA).

Statistical analysis

Statistical analysis was performed with the two-tailed *t*-test. Differences with $p < 0.05$ and $p < 0.01$ values were regarded as statistically significant and very significant, respectively.

RESULTS

APAP-induced ATP depletion correlated with down-regulation of AMPK activation

It is well known that APAP-mediated hepatotoxicity causes depletion of hepatic ATP levels by inhibiting mitochondrial function (Jaeschke and Bajt, 2006). In addition, it has been shown that stimulation of AMPK can increase the ATP level in several tissues (Hardie and Sakamoto, 2006). Therefore, we hypothesized that ATP depletion might be caused by the down-regulation of AMPK activation. Male C57BL/6N mice were treated with a single dose of APAP (400 mg/kg body weight) and the relationship between ATP levels and AMPK phos-

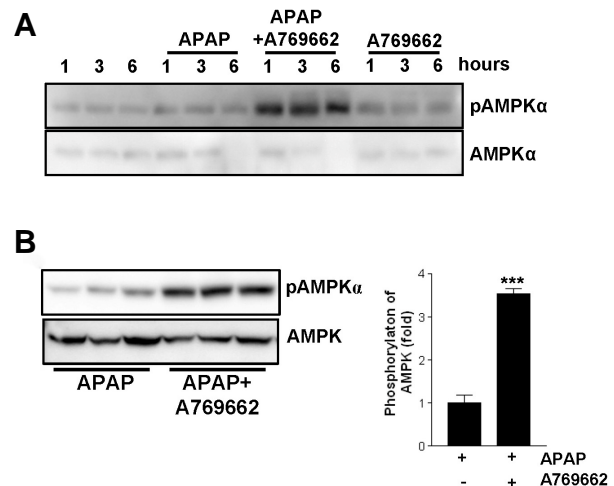


Fig. 2. Synergistic effects of A769662 and acetaminophen (APAP) on phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) in primary hepatocytes and liver tissues. (A) Primary hepatocytes were isolated from C57BL/6N male mice and cultured overnight. Cells were treated with A769662 (10 μ M) in the presence or absence of APAP (10 mM) for the indicated times, and time-dependent phosphorylation of AMPK α was determined by immunoblot analysis. (B) *In vivo* AMPK α phosphorylation was estimated in liver lysates from mice treated with APAP alone or APAP plus A769662 for 6 h. The bar chart shows the quantitative analysis of the ratio of pAMPK/total jun-N-terminal kinase (JNK) of each group; $n = 6$, $***P < 0.001$ as compared with APAP alone.

phorylation was estimated. Following APAP administration, ATP loss occurred rapidly while recovery was slow (Fig. 1A). Interestingly, active phosphorylation of AMPK declined at the same time point and with a similar pattern (Fig. 1B), suggesting that ATP depletion by APAP might correlate with inhibition of AMPK activity.

A769662 and APAP synergistically induced AMPK activity

Next, we evaluated whether A769662-induced activation of AMPK could ameliorate APAP-induced acute liver injury. Both A769662 and APAP administered alone, slightly enhanced AMPK phosphorylation in primary hepatocytes, as compared to the untreated control (Fig. 2A). However, co-treatment with A769662 and APAP led to strong and prolonged activation of AMPK for up to 6 h as compared to the other treated groups (Fig. 2A). Consistent with the *in vitro* study, a dramatic increase in AMPK activity was also observed with lysates from liver tissue samples treated with APAP and A769662 as compared to APAP alone (Fig. 2B).

A769662 prevented ATP loss and eliminated the toxic effects of APAP in liver tissues

Activation of AMPK stimulates ATP synthesis by enhancing the catabolic pathway. Therefore, we estimated the ATP levels in lysates from mice treated with 400 mg/kg of APAP for 6 h, in combination with or without A769662. Interestingly, A769662 treatment led to significantly elevated ATP levels as compared to APAP alone (Fig. 3A)

We then investigated the impact of A769662 on APAP-induced liver injury. As expected, serum levels of the established markers of liver injury, ALT and AST, were increased in mice 6 h following

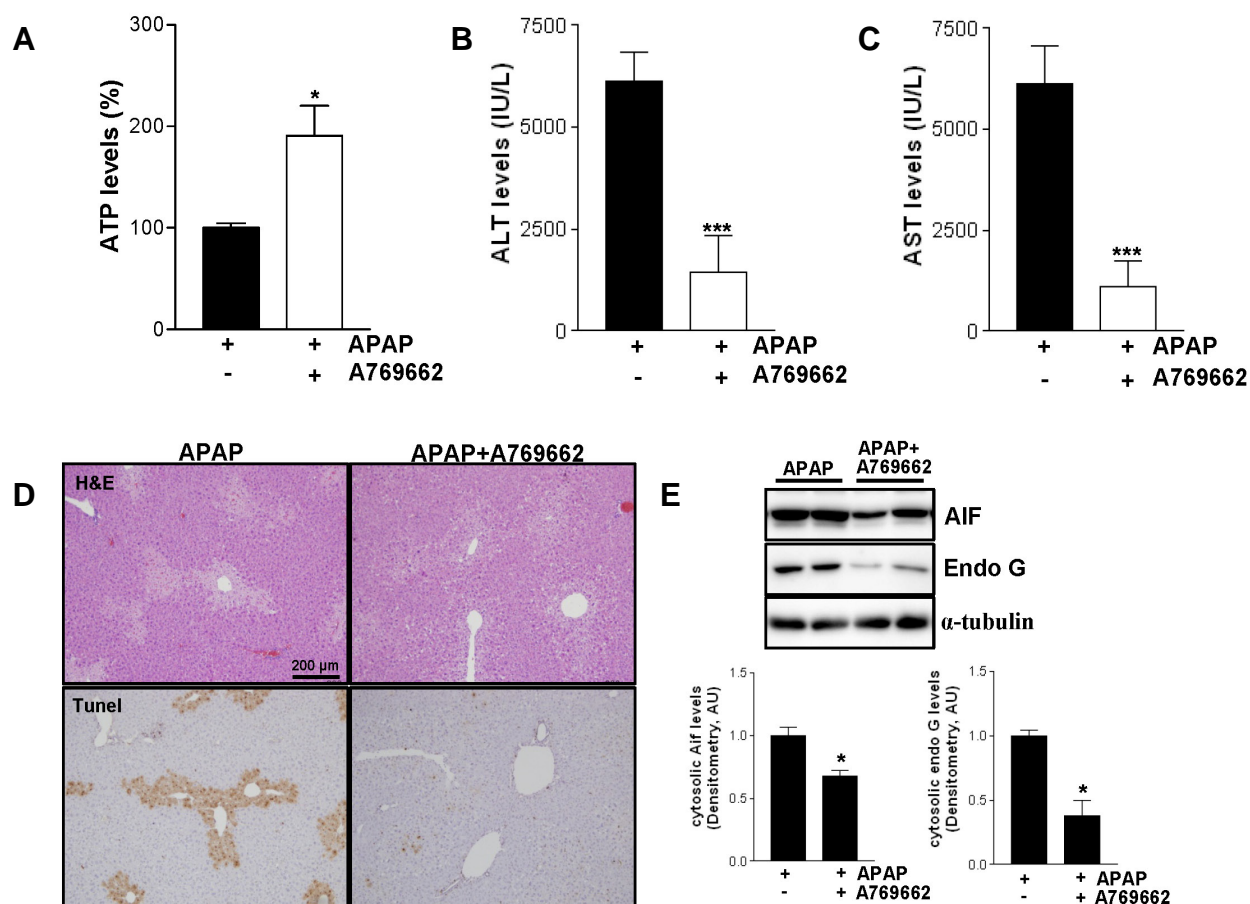


Fig. 3. Protective effects of A769662 against acetaminophen (APAP)-induced liver injury. Eight-week-old C57BL/6N male mice were intraperitoneally pretreated with A769662 (30 mg/kg) or vehicle 30 min before treatment with APAP (400 mg/kg). (A) Adenosine triphosphate (ATP) levels in the liver tissue samples were measured 6 h following APAP treatment. The data are expressed by % of APAP-treated group. (B) Plasma alanine aminotransferase (ALT) and (C) aspartate aminotransferase (AST) levels were compared between the APAP- and APAP plus A769662-treated groups (n = 6). (D) Histopathology was estimated by using hematoxylin and eosin (H&E, upper panel, $\times 100$) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL, lower panel, $\times 100$) staining. Bar indicates 200 μ m. (E) Immunoblots for the markers of cell death (apoptosis inducing factor, AIF and endonuclease G, endo G) in liver lysates from the APAP- or APAP plus A769662-treated groups are shown, n = 5-6, * $P < 0.05$, *** $P < 0.001$ as compared with APAP alone.

APAP treatment (Figs. 3B and 3C). H&E and TUNEL staining also revealed the presence of typical bridging necrosis within the centrilobular region of the liver sections from the APAP-treated mice (Fig. 3D). However, APAP-induced upregulation of ALT and AST, as well as the typical necrotic areas were markedly reduced by treatment with A769662 (Fig. 3D). Importantly, the cytosolic release of AIF and endo G induced by APAP were also recovered by A769662 administration (Fig. 3E). These data suggest that prevention of APAP-induced ATP loss by activation of AMPK has beneficial effects.

A769662 did not affect GSH level, JNK and extracellular signal-regulated kinase (ERK) activities, and nitrotyrosine formation mediated by APAP

Necrotic cell death induced by APAP is caused by complex factors, including GSH depletion, sustained JNK activation, and covalent binding (Jaeschke and Bajt, 2006; McGill et al., 2012). therefore, we examined whether these factors are involved in the A769662-mediated protection against APAP-induced

cytotoxicity. Hepatic GSH concentration showed a reduction of approximately 30% following APAP but not A769662 treatment (Fig. 4A). Unexpectedly, co-treatment with A769662 and APAP caused a greater reduction in GSH than APAP alone (Fig. 4B). Next, we investigated the phosphorylation of JNK and ERK, which are important molecules involved in APAP cytotoxicity. Significant differences in the phosphorylation of JNK1/2 and ERK1/2 were not observed between APAP-treated and APAP plus A769662-treated mice (Fig. 4B). Nitrotyrosine formation also was not different between APAP-treated and APAP plus A769662-treated mice (Fig. 4C). These data suggest that, the protective effects of A769662 against APAP-induced liver injury might be linked to a later step in the signal pathway after nitrotyrosine formation.

A769662 did not affect mitochondrial dysfunction induced by APAP

It is well known that activation of AMPK stimulates ATP synthesis by turning on the catabolic pathway. It is important to

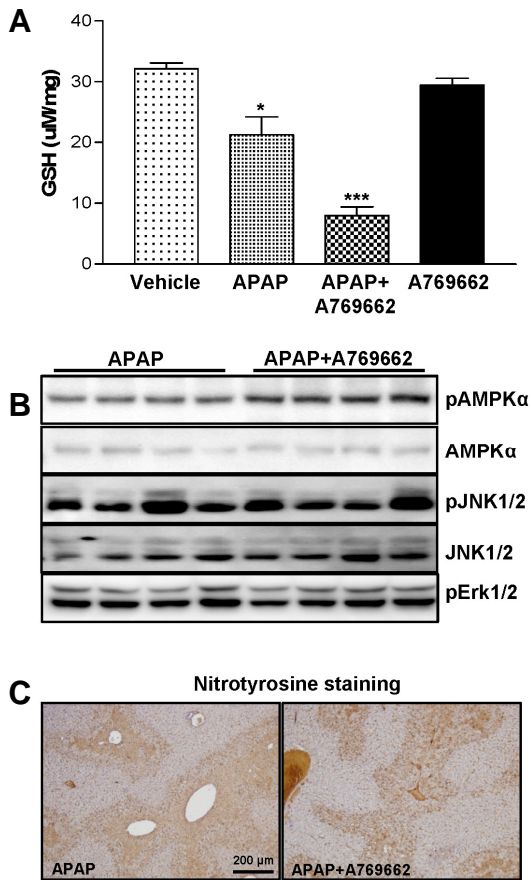


Fig. 4. Effects of A769662 on acetaminophen (APAP)-induced changes in glutathione (GSH), jun-N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) levels, and nitrotyrosine response in the liver. (A) GSH levels were measured in the indicated groups by enzymatic analysis 6 h following APAP treatment. (B) Activation of JNK, adenosine monophosphate-activated protein kinase (AMPK), and ERK1/2 in the liver following APAP treatment was analyzed by immunoblot by using antibodies (Abs) to detect phosphorylation. (C) Nitrotyrosine formation was determined by immunostaining by using an antibody against nitrotyrosine, and representative images were displayed. Bar indicates 200 µm, n = 5-6, * $P < 0.05$, *** $P < 0.001$ as compared to the vehicle.

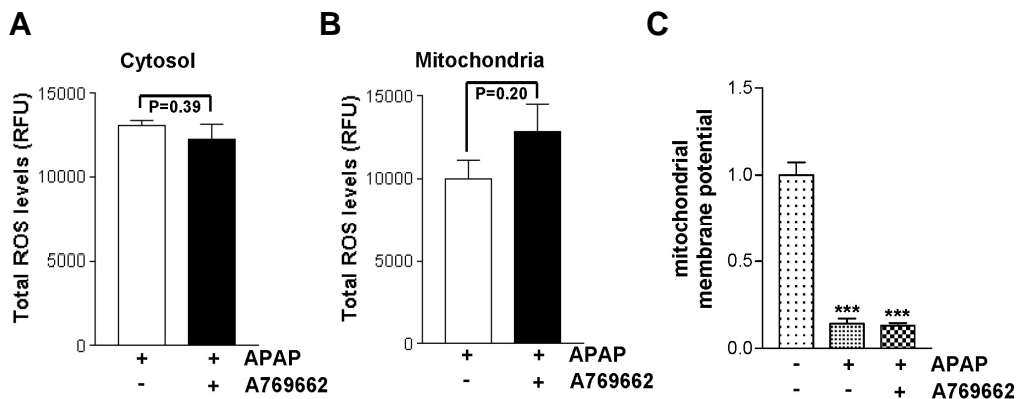


Fig. 5. Effects of A769662 on acetaminophen (APAP)-induced mitochondrial dysfunction. Total reactive oxygen species (ROS) production in cytosolic (A) and mitochondrial (B) fractions of the liver from APAP- or APAP plus A769662-treated mice (n = 5) measured as the oxidative conversion of 2',7'-dichlorofluorescein diacetate (H₂DCFDA) to highly fluorescent 2',7'-dichlorofluorescein (DCF). (C) Primary hepatocytes were treated with A769662 with or without 10 mM APAP for 12 h and mitochondrial membrane potential was calculated as the ratio of red (excitation 550 nm, emission 590 nm) to green fluorescence (excitation 485 nm, emission 535 nm) of JC-1 dye.

investigate mitochondrial function since most ATP is synthesized by the electron transport chain in the mitochondria. APAP overdose triggers mitochondrial dysfunction by producing ROS, which in turn causes the collapse of membrane potential and

necrotic cell death (Packer et al., 1997; Saito et al., 2010). ROS levels were slightly elevated in mitochondrial fractions from mice co-treated with A769662 and APAP as compared to APAP treatment alone (Figs. 5A and 5B). We also estimated the

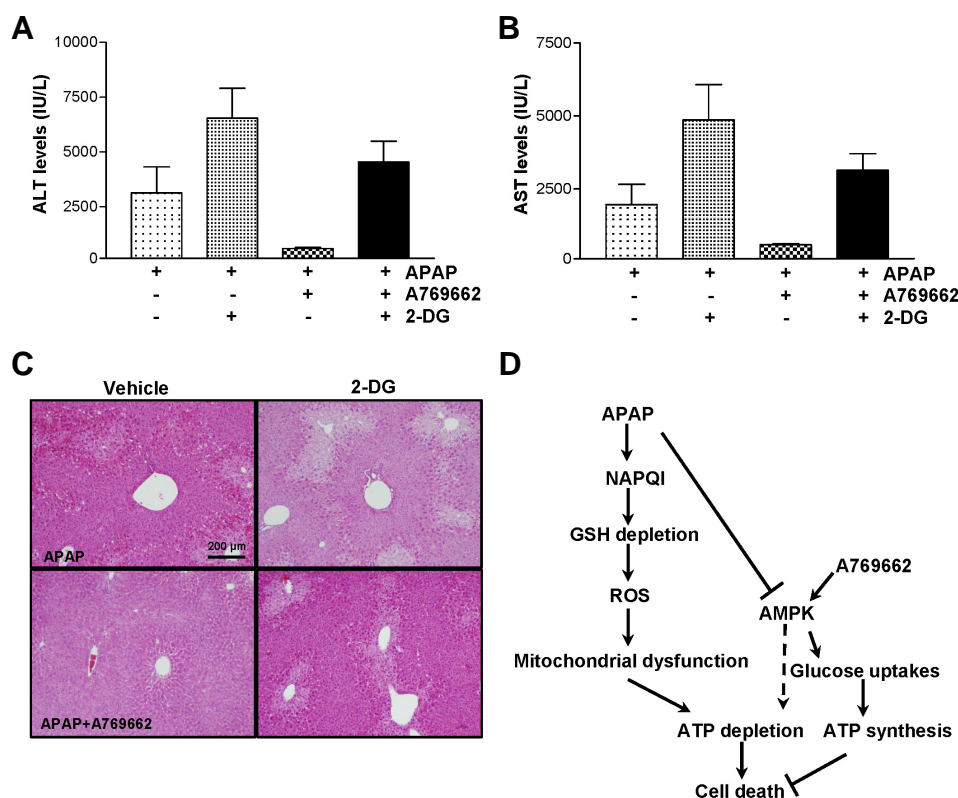


Fig. 6. Effects of inhibition of glycolysis by 2-deoxy-glucose (2DG) on beneficial effects of A769662 against acetaminophen (APAP)-induced liver injury. Male mice were intraperitoneally treated with A769662 (30 mg/kg) 30 min before APAP (400 mg/kg) and 2DG (500 mg/kg). After 6 h, serum (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) were measured automatically. (C) Liver sections were stained with hematoxylin and eosin (H&E). (D) Schematic illustration of our hypothesis.

effects of A769662 on APAP-induced mitochondrial dysfunction by JC-1 staining. APAP treatment significantly down-regulated mitochondrial membrane potential (Fig. 5C). A769662 did not reverse the loss in mitochondrial membrane potential caused by APAP in primary hepatocytes (Fig. 5C). These results suggest that a mitochondrial-independent ATP production pathway is responsible for the protective effects of A769662 against APAP hepatotoxicity.

Beneficial effects of A769662 on APAP-induced liver injury are completely eliminated by 2DG treatment

Most ATP synthesized in the mitochondria is through oxidative phosphorylation of substrates like glucose and free fatty acid; however, ATP is also produced in the cytosol during glycolysis (Viollet et al., 2009). A769662 does not prevent APAP-induced mitochondrial dysfunction and may induce ATP synthesis via increased glycolysis. To investigate this phenomenon, we inhibited glycolysis with 2DG as previously reported (Qian et al., 1999). Excitingly, 2DG treatment eliminated the beneficial effects of A769662 on plasma ALT and AST levels (Figs. 6A and 6B). Histological data also showed similar results (Fig. 6C), suggesting that the therapeutic effects of A769662 against APAP-induced liver injury are associated with increased ATP production through stimulation of glycolysis (shown in the schematic Fig. 6D).

DISCUSSION

APAP overdose induces necrotic cell death, which correlates with ATP loss caused by severe mitochondrial dysfunction (Hinson et al., 2010; Hwang et al., 2014; Jaeschke and Bajt, 2006). In this study, we observed that the pharmacological activation of AMPK significantly ameliorates APAP-induced liver

injury. Co-treatment with A769662 and APAP strongly enhanced AMPK phosphorylation and prevented the ATP loss induced by APAP. However, APAP-induced mitochondrial dysfunction, a major source of ATP generation, was not improved by treatment with A769662. Interestingly, inhibition of glycolysis by 2DG caused the complete elimination of the beneficial effects of AMPK activation against APAP-induced liver injury, suggesting that ATP loss can affect susceptibility to APAP-induced liver toxicity.

Energy homeostasis is a critical factor in cell viability. APAP-induced hepatotoxicity leads to ATP depletion, which results in cellular swelling, as well as rounding and swelling of mitochondria and plasma membrane protrusions (Hinson et al., 2010). Consistently, the current study also showed a rapid loss of ATP following APAP treatment in a mouse model. In the current study, we observed that APAP treatment decreases active phosphorylation of the Thr172 active site of AMPK, following a pattern similar to that seen with ATP loss. Eukaryotic cells use AMPK as a mechanism to sense cellular ATP concentration (Hardie and Sakamoto, 2006). AMPK activation upregulates ATP-producing catabolic pathways involving fatty acid oxidation and glycolysis and inhibits ATP-consuming pathways involving synthesis of fatty acids, cholesterol, glycogen, and proteins (Mihaylova and Shaw, 2011; Yang et al., 2010). In addition to its ability to restore energy, AMPK activation inhibits apoptosis by decreasing ROS in several types of cells (Dong et al., 2014; Qiu et al., 2011). Jia et al. suggested that AMPK activation decreases the effects of homocysteine which increases oxidative stress and apoptosis in endothelial progenitor cells (Jia et al., 2011). Previous studies have provided that PI3K/PKB and ERK signaling pathways and endoplasmic reticulum stress are involved in the anti-apoptotic effects of AMPK activation in neutrophils (Rossi and Lord, 2013) and endothelial cells (Jia et al.,

2012). However, A769662 treatment didn't inhibit APAP-induced ROS induction and show any differences in the ERK signaling pathways. These findings suggest that other pathways might be associated with the anti-apoptotic effects of A769662-mediated AMPK activation. Another study suggests that AMPK may be involved in the APAP-induced ATP loss in the liver (Saberri et al., 2014). They also suggested that PKC activation response to APAP caused inactivation of AMPK. However, more work need to know how APAP regulate AMPK activity (Saberri et al., 2014).

Over a period of several decades, a series of beneficial compounds with the ability to activate AMPK have been identified and tested in the clinical setting (Fogarty and Hardie, 2010; Steinberg and Kemp, 2009). We used one of these compounds, A769662, as an AMPK activator for ATP production. A769662 is a thienopyridine derivative known to directly interact with AMPK and stimulate its activation by distinct molecular mechanisms (Cool et al., 2006; Goransson et al., 2007; Sanders et al., 2007). In our study, A769662 in combination with APAP strongly stimulated AMPK activity *in vitro* and *in vivo* and showed protective effects against APAP-induced ATP loss. Furthermore, co-treatment with A769662 and APAP significantly protected mice against APAP-induced liver injury as evidenced by reduced plasma ALT and AST levels as well as histological signs of cell death. A previous study also suggested that 15-20% of the normal ATP level is sufficient to suppress the necrotic cell death in the hepatic ischemia/reperfusion injury model (Nieminen et al., 1994). Our data showed that the stimulation of ATP production might be effective in clinical trials in the treatment of patients with drug-induced liver injury.

APAP-induced hepatotoxicity is initiated by the formation of the toxic metabolite NAPQI by cytochrome P450. NAPQI causes glutathione depletion, oxidative stress, formation of nitrotyrosine by mitochondrial proteins, loss of mitochondrial membrane potential (MMP), and formation of the mitochondrial permeability transition (MPT) pore (Hardie and Sakamoto, 2006; Hinson et al., 2010). The loss of MMP causes ATP depletion. Several studies have suggested that the chemical activation of AMPK inhibits apoptosis and restores MMP and have provided evidence that cell viability depends on mitochondrial function (Shin et al., 2009; Stefanelli et al., 1998). In contrast with other studies (Shin et al., 2009; Stefanelli et al., 1998), we did not detect any significant association in these parameters between mice treated with APAP alone and those co-treated with APAP and A769662. Indeed, ROS production response to APAP was not inhibited by treatment of A769662. GSH levels were more diminished by addition of A769662 than APAP alone as shown in Fig. 4. JC-1 staining also showed that A769662 treatment did not improve the mitochondrial dysfunction mediated by APAP. These results suggest that the A769662-mediated prevention of APAP-induced loss of ATP, which occurred in a mitochondrial-independent manner. Glycolysis is another pathway by which ATP is generated (Jung et al., 2013; Viollet et al., 2009). Previous studies showed the importance of ATP generation by glycolysis, in an ischemia/reperfusion injury model (Qian et al., 1999). This study revealed that the availability of the glycolytic substrate prevented significant ATP depletion and cellular necrosis (Qian et al., 1999). Similarly, we also observed that the inhibition of the glycolytic pathway by 2DG completely eliminated the beneficial effects of A769662 against APAP-induced liver injury. This result suggests the importance of glycolysis during APAP-induced liver injury.

In conclusion, we described for the first time that the activation of AMPK by A769662 provides protection against APAP

hepatotoxicity. The beneficial effects of A769662 on APAP-induced acute liver injury did not extend to several pathological symptoms, including reduced GSH levels, sustained JNK activation, nitrotyrosine formation, enhanced ROS, and mitochondrial dysfunction. We also observed that inhibition of glycolysis by 2DG eliminated the protective effects of A769662. This result suggests that ATP synthesis by glycolysis is important for the A769662-mediated protection against APAP-induced liver injury. Finally, our study shows that the ATP loss is a crucial causing factor in the APAP-induced liver injury and targeting of ATP production pathway such as AMPK may be considered a potentially new approach for the treatment of patients with drug-induced liver injury.

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REFERENCES

- Cohen, S.D., and Khairallah, E.A. (1997). Selective protein arylation and acetaminophen-induced hepatotoxicity. *Drug Metab. Rev.* 29, 59-77.
- Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., Dickinson, R., Adler, A., Gagne, G., Iyengar, R., et al. (2006). Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* 3, 403-416.
- Dahlin, D.C., Miwa, G.T., Lu, A.Y., and Nelson, S.D. (1984). N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc. Natl. Acad. Sci. USA* 81, 1327-1331.
- Dong, G.Z., Lee, J.H., Ki, S.H., Yang, J.H., Cho, I.J., Kang, S.H., Zhao, R.J., Kim, S.C., and Kim, Y.W., (2014). AMPK activation by isorhamnetin protects hepatocytes against oxidative stress and mitochondrial dysfunction. *Eur. J. Pharmacol.* 740, 634-640.
- Fogarty, S., and Hardie, D.G. (2010). Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. *Biochim. Biophys. Acta* 1804, 581-591.
- Goransson, O., McBride, A., Hawley, S.A., Ross, F.A., Shpiro, N., Foretz, M., Viollet, B., Hardie, D.G., and Sakamoto, K. (2007). Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *J. Biol. Chem.* 282, 32549-32560.
- Hardie, D.G., and Sakamoto, K. (2006). AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology* 21, 48-60.
- Hinson, J.A., Roberts, D.W., and James, L.P. (2010). Mechanisms of acetaminophen-induced liver necrosis. *Handb. Exp. Pharmacol.* 2010, 369-405.
- Hwang, J.H., Kim, Y.H., Noh, J.R., Gang, G.T., Kim, K.S., Chung, H.K., Tadi, S., Yim, Y.H., Shong, M., and Lee, C.H. (2014). The protective role of NAD(P)H:quinone oxidoreductase 1 on acetaminophen-induced liver injury is associated with prevention of adenosine triphosphate depletion and improvement of mitochondrial dysfunction. *Arch. Toxicol.* [Epub ahead of print].
- Ido, Y., Carling, D., and Ruderman, N. (2002). Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes* 51, 159-167.
- Jaesckhe, H., and Bajt, M.L. (2006). Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol. Sci.* 89, 31-41.
- Jia, F., Wu, C., Chen, Z., Lu, G. (2011). AMP-activated protein kinase inhibits homocysteine-induced dysfunction and apoptosis in endothelial progenitor cells. *Cardiovasc. Drugs Ther.* 25, 21-25.
- Jia, F., Wu, C., Chen, Z., Lu, G. (2012). Atorvastatin inhibits homocysteine-induced endoplasmic reticulum stress through activation of AMP-activated protein kinase. *Cardiovasc. Ther.* 30,

- 317-325.
- Jung, Y., Jang, Y.J., Kang, M.H., Park, Y.S., Oh, S.J., Lee, D.C., Xie, Z., Yoo, H.S., Park, K. C., Yeom, Y.I., (2013). Metabolic signature genes associated with susceptibility to pyruvate kinase, muscle type 2 gene ablation in cancer cells. *Mol. Cells* 35, 335-341.
- Larson, A.M., Polson, J., Fontana, R.J., Davern, T.J., Lalani, E., Hynan, L.S., Reisch, J.S., Schiodt, F.V., Ostapowicz, G., Shakil, A.O., et al. (2005). Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. *Hepatology* 42, 1364-1372.
- McGill, M.R., Williams, C.D., Xie, Y., Ramachandran, A., and Jaeschke, H. (2012). Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. *Toxicol. Appl. Pharmacol.* 264, 387-394.
- Mihaylova, M.M., and Shaw, R.J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat. Cell Biol.* 13, 1016-1023.
- Nelson, S.D. (1990). Molecular mechanisms of the hepatotoxicity caused by acetaminophen. *Semin. Liver Dis.* 10, 267-278.
- Nieminen, A.L., Saylor, A.K., Herman, B., and Lemasters, J.J. (1994). ATP depletion rather than mitochondrial depolarization mediates hepatocyte killing after metabolic inhibition. *Am. J. Physiol.* 267, C67-74.
- Packer, M.A., Scarlett, J.L., Martin, S.W., and Murphy, M.P. (1997). Induction of the mitochondrial permeability transition by peroxynitrite. *Biochem. Soc. Trans.* 25, 909-914.
- Peralta, C., Bartrons, R., Serafin, A., Blazquez, C., Guzman, M., Prats, N., Xaus, C., Cutillas, B., Gelpi, E., and Rosello-Catafau, J. (2001). Adenosine monophosphate-activated protein kinase mediates the protective effects of ischemic preconditioning on hepatic ischemia-reperfusion injury in the rat. *Hepatology* 34, 1164-1173.
- Polson, J., and Lee, W.M. (2005). AASLD position paper: the management of acute liver failure. *Hepatology* 41, 1179-1197.
- Qian, T., Herman, B., and Lemasters, J.J. (1999). The mitochondrial permeability transition mediates both necrotic and apoptotic death of hepatocytes exposed to Br-A23187. *Toxicol. Appl. Pharmacol.* 154, 117-125.
- Qiu, G., Wan, R., Hu, J., Mattson, M.P., Spangler, E., Liu, S., Liu, S., Yau, S.-Y., Lee, T.M.C., Gleichmann, M., et al. (2011). Adiponectin protects rat hippocampal neurons against excitotoxicity. *Age* 33, 155-165.
- Rossi, A., and Lord, J.M. (2013). Adiponectin inhibits neutrophil apoptosis via activation of AMP kinase, PKB and ERK 1/2 MAP kinase. *Apoptosis* 18, 1469-1480.
- Saberi, B., Ybanez, M.D., Johnson, H.S., Gaarde, W.A., Han, D., and Kaplowitz, N. (2014). Protein kinase C (PKC) participates in acetaminophen hepatotoxicity through c-jun-N-terminal kinase (JNK)-dependent and -independent signaling pathways. *Hepatology* 59, 1543-1554.
- Saito, C., Lemasters, J.J., and Jaeschke, H. (2010). c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. *Toxicol. Appl. Pharmacol.* 246, 8-17.
- Sanders, M.J., Ali, Z.S., Hegarty, B.D., Heath, R., Snowden, M.A., and Carling, D. (2007). Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. *J. Biol. Chem.* 282, 32539-32548.
- Shin, S.M., Cho, I.J., and Kim, S.G. (2009). Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycogen synthase kinase-3beta inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway. *Mol. Pharmacol.* 76, 884-895.
- Stefanelli, C., Stanic, I., Bonavita, F., Flamigni, F., Pignatti, C., Guarneri, C., and Calderera, C.M. (1998). Inhibition of glucocorticoid-induced apoptosis with 5-aminoimidazole-4-carboxamide ribonucleoside, a cell-permeable activator of AMP-activated protein kinase. *Biochem. Biophys. Res. Commun.* 243, 821-826.
- Steinberg, G.R., and Kemp, B.E. (2009). AMPK in health and disease. *Physiol. Rev.* 89, 1025-1078.
- Towler, M.C., and Hardie, D.G. (2007). AMP-activated protein kinase in metabolic control and insulin signaling. *Circ. Res.* 100, 328-341.
- Viollet, B., Athes, Y., Mounier, R., Guigas, B., Zarrinpashneh, E., Horman, S., Lantier, L., Hebrard, S., Devin-Leclerc, J., Beauloye, C., et al. (2009). AMPK: Lessons from transgenic and knockout animals. *Front Biosci.* 14, 19-44.
- Yang, Y.M., Han, C.Y., Kim, Y.J., and Kim, S.G. (2010). AMPK-associated signaling to bridge the gap between fuel metabolism and hepatocyte viability. *World J. Gastroenterol.* 16, 3731-3742.