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Rifampicin activates AMPK and alleviates oxidative stress in the liver as mediated with Nrf2 signaling

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ABSTRACT

Although rifampicin could have a hepatic toxic effect, it has also been shown that this chemical acts as a cellular protectant against oxidative stress. Therefore, we wondered whether rifampicin has a beneficial effect such as an anti-oxidant in the liver, because the efficacy of some drugs sometimes relates with their toxicity as well as protective effects. The present study aimed to investigate the antioxidant effect of rifampicin against arachidonic acid (AA) plus iron (AA + iron) cotreatment and against acetaminophen (APAP, 500 mg/kg)-induced oxidative stress, *in vitro* and *in vivo*, respectively. *In vivo*, oral administration of rifampicin (100 or 200 mg/kg) attenuated elevation of serum alanine aminotransferase (ALT) and aspartate transaminase (AST), serum liver injury markers, against APAP treatment and, histologically, ameliorated tissue damage. Under *in vitro* examination, MTT assays were used to assess the cell death inhibitory effect of rifampicin against AA + iron-induced oxidative stress. In addition, DCFH-DA and Rh 123 staining showed that rifampicin treatment reduced reactive oxygen species (ROS) production and mitochondrial membrane damage, which had been induced by AA + iron treatment. Further, we explored whether rifampicin treatment enhanced phosphorylation of AMP-activated protein kinase (AMPK) by activation of liver kinase B1 (LKB1), the upstream kinase of AMPKα. Activated AMPKα induced activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), which are proteins functioning in redox balance. Moreover, we confirmed a reversed cell protective effect of rifampicin under compound C (an AMPK inhibitor) treatment. Overall, our data demonstrate that rifampicin effectively protects the liver against cellular oxidative stress through AMPKα and Nrf2 pathway.

1. Introduction

Reactive oxygen species (ROS) are more highly produced in the oxidative stress than the antioxidant capacity of cells [1,2]. Under normal cell conditions, a small amount of ROS is produced naturally during metabolism [3] and mitochondria provide a cellular defense by detoxifying the ROS [4]. However, when an overabundance of ROS is produced, it leads to cellular lipid, protein, DNA, and mitochondrial damage and can act as a carcinogen due to the role of ROS in mutagenesis and tumor growth [5,6]. ROS appear to be catalyzed in the presence of an excessive amount of iron, which is mostly located in liver tissue. Over-production of ROS can cause liver inflammation and progressive liver inflammation promotes the production of interleukins 1 and 6, which act as fibroblast growth factors, by Kupffer cells. Such cytokine production results in accumulation of connective tissue from fibroblasts due to a prolonged wound healing process [7,8]. Progressive fibrosis of liver tissue may result in liver fibrosis and cirrhosis, finally leading to hepatocellular carcinoma (HCC), which has a high mortality rate worldwide [9]. As the liver is the main organ associated with detoxification, oxidative stress is considered as one of the major causes of liver pathogenesis [10].

In vitro, we used arachidonic acid (AA), which is a ω-6 polyunsaturated fatty acid, as an oxidative stress causative agent. AA is released during oxidation of fatty acids and phospholipids and acts as a pro-inflammatory agent, further inducing apoptosis [11]. Under the presence of excessive iron, AA increases the production of ROS and promotes mitochondrial dysfunction [12]. Numerous papers have used AA plus iron (AA + iron) cotreatment and reported it to be an excellent oxidative stress inducer [12–14]. *In vivo*, acetaminophen (APAP) can be used as an oxidative stress-inducing liver injury agent. APAP is a

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Abbreviations

AA	arachidonic acid
ACC	acetyl-coenzyme A carboxylase
ALT	alanine aminotransferase
AMPK	AMP-activated protein kinase
APAP	acetaminophen
AST	aspartate aminotransferase
DCFH-DA	dichlorofluorescein diacetate
DMSO	dimethyl sulfoxide
GSH	glutathione
HCC	hepatocellular carcinoma
HE	hematoxylin and eosin
HO-1	heme oxygenase-1
IHC	immunohistochemistry
Keap1	Kelch-like ECH-associated protein 1
LKB1	liver kinase B 1
MMP	mitochondria membrane permeability
MPTP	mitochondrial permeability transition pore
MTT	3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide
NAPQI	N-acetyl-p-benzoquinone imine
NQO-1	NAD(P)H quinine oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
PARP	Poly (ADP-ribose) polymerase
PXR	pregnane X receptor
Rh 123	rhodamine 123
RIF	rifampicin
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

commonly used analgesic and antipyretic drug, and, in overdose, it promotes necrosis of hepatocytes adjacent to the central vein in liver [15]. In various previous studies, APAP overdose treatment has been used as an acute liver failure model in animal experiments [16–18]. APAP binds to glutathione (GSH), causing the depletion of GSH and creating a loss of antioxidant effect and accumulation of ROS [19]. P450 enzymes (mainly CYP2E1) oxidize APAP to form reactive N-acetyl-p-benzoquinone imine (NAPQI) and deplete GSH, leading to the binding of NAPQI to proteins and causing protein damage [20]. Therefore, in the present study, we used cotreatment of (AA) + iron in cells and APAP in animals to cause oxidative stress [13,21,22].

Under cellular energy stress conditions, AMP-activated protein kinase (AMPK) is activated by phosphorylation of threonine residue site [23,24]. Previous studies have reported that AMPK activation enhances cellular antioxidant capacity through nuclear localization of nuclear factor-erythroid 2 related factor 2 (Nrf2) [25–27]. Nrf2 activation is an important factor in the cell defense system. Nrf2 is a transcription factor that regulates multiple cytoprotective genes that encode proteins, and its functions include detoxifying toxins [27,28]. Here, rifampicin was expected to act as an antioxidant through AMPK and Nrf2 signaling pathways in hepatocytes.

The pregnane X receptor (PXR) is a regulator of genes that have roles in detoxification and elimination of xenobiotics in the body (19940802). Rifampicin is a well-known activator of PXR, is mainly used as an anti-tuberculosis or leprosy drug, and is reported to act as a neuroprotectant against oxidative stress [29–32]. On the other hand, rifampicin can be used as a liver toxicity inducer in combination, mostly, with isoniazid [33,34]. However, the effect of rifampicin on oxidative stress has not been fully elucidated.

In this present study, we investigated the hepatocyte protective effect of rifampicin *in vitro* and *in vivo* against AA + iron and APAP treatment, respectively. Rifampicin ameliorated tissue injury in an animal model against APAP. Moreover, we detected an underlying antioxidant mechanism of rifampicin. Rifampicin activated AMPKα and its downstream signals by LKB1 phosphorylation. In addition, the activated AMPKα induced nuclear localization of Nrf2, elevating heme oxygenase-1 (HO-1) expression. Our observations indicate that the antioxidant effect of rifampicin acts through AMPKα-mediated Nrf2 signaling pathways.

2. Material and methods

2.1. Reagent

Rifampicin, acetaminophen (APAP), ferric nitrate (Fe, iron), 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide (MTT), rhodamine 123 (Rh 123), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), Harri's hematoxylin, and eosin were purchased from Sigma (St. Louis, MO, USA). AA was obtained from Calbiochem (San diego, CA, USA). Anti-poly (ADP-ribose) polymerase (PARP), anti-caspase3, anti-β-actin, anti-phospho-ACC, anti-phospho-AMPKα, anti-phospho-liver kinase B1 (LKB1), anti-heme oxygenase-1 (HO-1), anti-nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and anti-lamin a/c antibodies were supplied from Cell Signaling Technology (Danvers, MA, USA).

2.2. Animals and treatment

Male ICR mice (5 weeks old) were purchased from Charles River Orient Bio (Seongnam, Korea). *In vivo* experiment acquired approval from the Institutional Animal Care and Use Committee of the Daegu Haany University and conducted in agreement with the guidelines of the National Institutes of Health. Rifampicin was dissolved in 40% polyethylene glycol (PEG) and orally administered for three consecutive days (100 mg/kg and 200 mg/kg). Followed by last administration of rifampicin, 500 mg/kg APAP was orally injected after 16 h starvation. All mice were sacrificed 6 h after APAP injection, blood and liver tissue samples were collected. To separate serum, whole blood was incubated at room temperature for 2 h and centrifuged for 5 min at 5000 rpm [22].

2.3. Harri's hematoxylin and eosin (HE) stain

Formalin fixed liver tissue were made into paraffin block by passing tissue processing and embedding procedures. Hydrolyzed sections were stained in hematoxylin for 10 min. Followed by bluing in ammonia water, sections were stained with eosin for 10 s. After stain, tissue sections were dehydrated and cleared prior to mounting. Histological changes were observed under light microscope (Nikon, Tokyo, Japan) [22].

2.4. Immunohistochemistry (IHC)

A BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) was used in IHC. 4 μm thick liver tissue sections on coated glass slides and heated at 60 °C for 2 h. EZ prep solution (Ventana Medical Systems) was used to deparaffinize followed by CC1 solution. CC1 solution (Ventana Medical Systems) was used as heat-induced antigen retrieval agent. Anti-p-AMPKα antibody was diluted to 1:100 and applied on slides manually and incubated for 60 min. Universal DAB detection kit (Ventana Medical Systems) was used to detect specific reaction and nucleus was stained with hematoxylin followed by bluing reagent.

2.5. Cell culture

HepG2, SK-Hep-1 and Huh-7 cells obtained from American Type Culture Collection (ATCC). HepG2 cells were maintained Dulbecco's modified Eagle's medium liquid (DMEM) low glucose with 10% FBS and 10 mg/ml normocin. SK-Hep-1 and Huh-7 cells were maintained in DMEM high glucose with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.6. MTT assay

To detect cell viability, HepG2 cells were seeded in a 48-well plate at a density of 1×10^5 cells per well [13]. Cells were starved for 12 h in FBS free DMEM low glucose medium followed by different doses (3, 10, 30 μM) of rifampicin treatment 1 h prior to 10 μM AA treatment. After 12 h of incubation, cells were exposed to 5 μM iron for 2 h [22]. Cell viability was defined relative to untreated control.

2.7. Immunoblot analysis

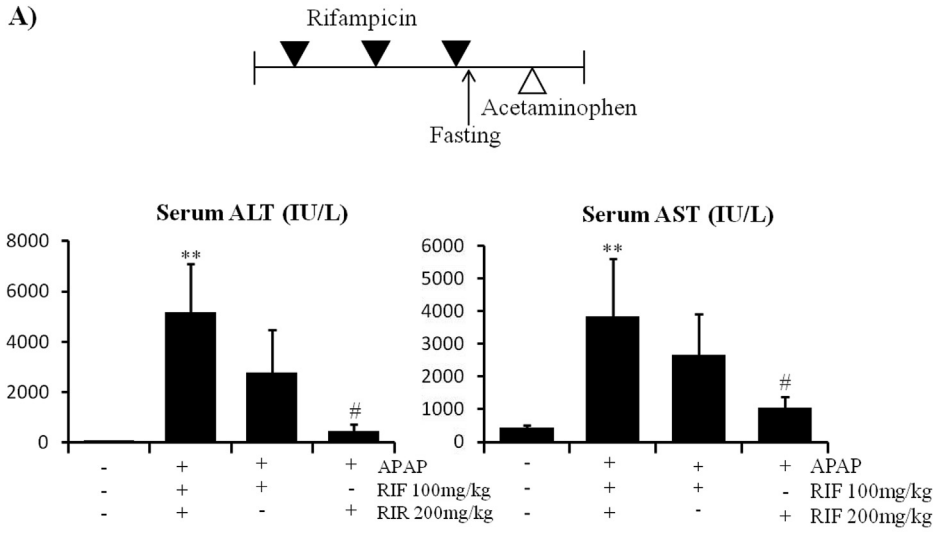
Treated cells as described were lysed using RIPA buffer at 4 °C [13]. The bands were developed using ECL reagent (Advensta, Menlo Park, CA, USA) and a chemi-doc image analyzer (Vilber Lourmat, France).

2.8. Nuclear extraction

HepG2 cells were plated in 6-well plate and treat with rifampicin in different time and doses as described [13,26]. Cells were lysed in hypotonic buffer for 10 min and then centrifuged at 7500 g for 10 min at 4 °C. The pellets were re-lysed in hypertonic buffer for 60 min and vortexed every 10 min. Supernatant was collected after centrifugation at 15,000 g for 15 min at 4 °C.

2.9. Reactive oxygen species (ROS) production measurement

Intracellular ROS production was measured by DCFH-DA staining [22]. Fluorescence intensity was measured by using ELISA microplate reader (Tecan, Research Triangle Park, NC, USA) at 485 nm of excitation and 535 nm of emission.



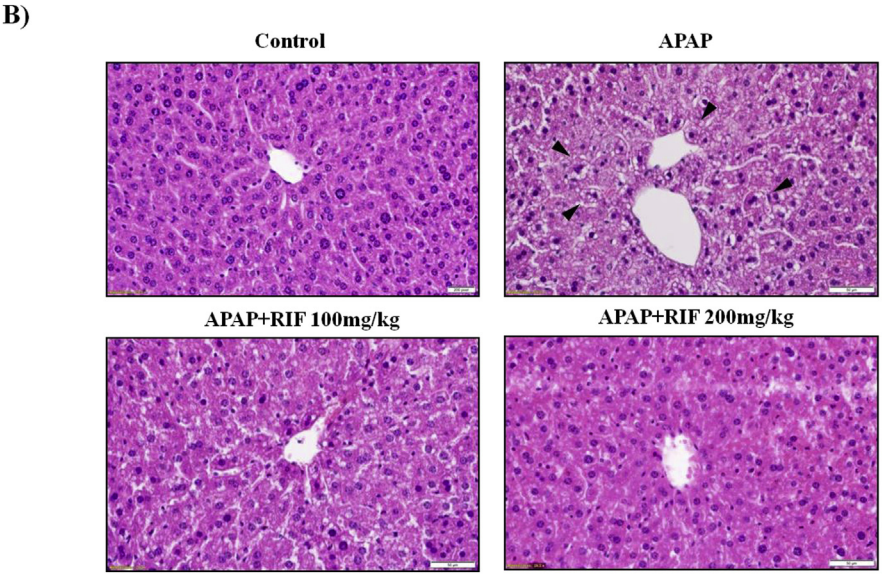


Fig. 1. Effect of rifampicin in acetaminophen-induced liver toxicity model.
(A) Effect of rifampicin on serum ALT and AST level. Mice were orally treated with 100 mg/kg or 200 mg/kg of rifampicin for three consecutive days, and 500 mg/kg of acetaminophen was orally injected 16 h starvation after last administration of rifampicin. (B) H&E staining. Liver tissues demonstrated the hepatocyte degeneration (arrows). Data represent the mean ± SD of eight mice (**$\rho < 0.01$ between vehicle and acetaminophen treated group; #$\rho < 0.05$ between acetaminophen treated group with or without rifampicin). Scale bars = 50 μm. APAP, acetaminophen; RIF, rifampicin.

2.10. Mitochondrial membrane potential (MMP) measurement

MMP was measured by flow cell analysis with Rh 123 staining [22]. HepG2 cells were plated at 3×10^7 per well and incubated in serum free media for 12 h. Rifampicin was treated as described and then, 10 μM Rh 123 was treated after iron stimulation for 1 h. Fluorescence was detected by BD Accuri C6 Plus Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.11. Statistical analysis

One-way analysis of variance procedures was used to assess significant differences among treatment groups. The criterion for statistical significance was set at $\rho < 0.05$ or $\rho < 0.01$.

3. Result

3.1. Effect of rifampicin on APAP-induced liver injury

We used APAP, a well-known liver injury inducer, to cause liver injury in a murine model in order to evaluate the efficacy of rifampicin against oxidative stress [35–37]. In our blood analysis, we measured the serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST), liver injury markers. As predicted, APAP significantly increased serum ALT and AST levels from those in the control groups (Fig. 1A). Pretreatment of rifampicin (100 mg/kg and 200 mg/kg) attenuated the elevation of serum ALT and AST levels (Fig. 1A) [38]. To determine the histological effects of APAP, we used H&E staining and light microscopy to examine mouse liver tissue sections. In the APAP-treated group, we detected swelling of hepatocytes, breakdown of the hepatocyte lining pattern, and shrinkage of nuclei. In the rifampicin-pretreated group, the drug produced inhibition of APAP-induced liver injuries. The histological results showed that the 100 mg/kg rifampicin treatment was less effective than the 200 mg/kg rifampicin treatment (Fig. 1B).

3.2. Effect of rifampicin on AA + iron-induced oxidative stress and mitochondrial damage

We used AA + iron, widely used as an oxidative stress causative agent [12,22,39], to induce oxidative stress *in vitro*. Initially, we investigated whether rifampicin had an apoptosis inhibitory effect against AA + iron treatment. In MTT assays, different rifampicin doses (3, 10, and 30 μM) were treated along with the AA + iron oxidative stress inducer, and the 30 μM rifampicin dose effectively protected against cell death (Fig. 2A). Therefore, the 30 μM rifampicin dosage was used in further experiments. Next, we performed immunoblotting to examine the expression levels of apoptosis-related protein markers, poly ADP-ribose polymerase (PARP) and procaspase 3. As expected, AA + iron stimulated cleavage of PARP and decreased procaspase 3 expression; however, the 30 μM rifampicin treatment inhibited the AA + iron-induced cleavage of PARP and loss of procaspase 3 expression (Fig. 2B).

Furthermore, we investigated the effect of rifampicin on the level of ROS produced by AA + iron. Intracellular ROS levels were measured by a spectrophotometer using dichloro-dihydro-fluorescein diacetate (DCFH-DA) [40,41]. The results showed that AA + iron-treated cells produced the increase in ROS level over that in the control cells, and the 30 μM rifampicin treatment potently attenuated the AA + iron-elevated ROS level (Fig. 3A).

Under redox system imbalance, oxidative stress promotes loss of mitochondrial membrane permeability and mitochondrial defense systems [42]. Therefore, we examined the protective effect of rifampicin on mitochondria. For that purpose, we used flow cytometric analysis using Rh 123, a lipophilic cationic dye that can detect changes in MMP [43]. The AA + iron-treated group increased the cell population of low Rh123 intensity (RN1 fraction) compared to that of the control group, indicating the presence of mitochondrial dysfunction and loss of MMP. However, 30 μM rifampicin significantly prevented the AA + iron-induced mitochondrial depolarization (Fig. 3B). These results indicate that rifampicin has a mitochondria protective effect.

3.3. Effect of rifampicin on AMPKα activation

In HepG2 cells, the 30 μM rifampicin activated LKB1, an upstream kinase of AMPKα, after 30 min of incubation. Activated LKB1 phosphorylates AMPKα as well as acetyl-coenzyme A carboxylase (ACC), a downstream target of AMPKα [44]. Following 3–6 h of incubation with rifampicin, we observed the highest expression of p-AMPKα (Fig. 4A). In other types of liver cell lines (*i.e.*, SK-Hep-1 and Huh-7 cells), the highest expression of p-AMPKα occurred after 10 min of incubation with 30 μM rifampicin (Fig. 4B). To confirm rifampicin-based mediation of the AMPKα in hepatocyte protective effect, we used HeLa cells, LKB1 deficient cells, and compound C, an AMPK inhibitor. The MTT assays showed that HeLa cells treated with AA + iron and rifampicin produced a partial opposite result from that for HepG2 cells treated with AA + iron and rifampicin (Fig. 4C).

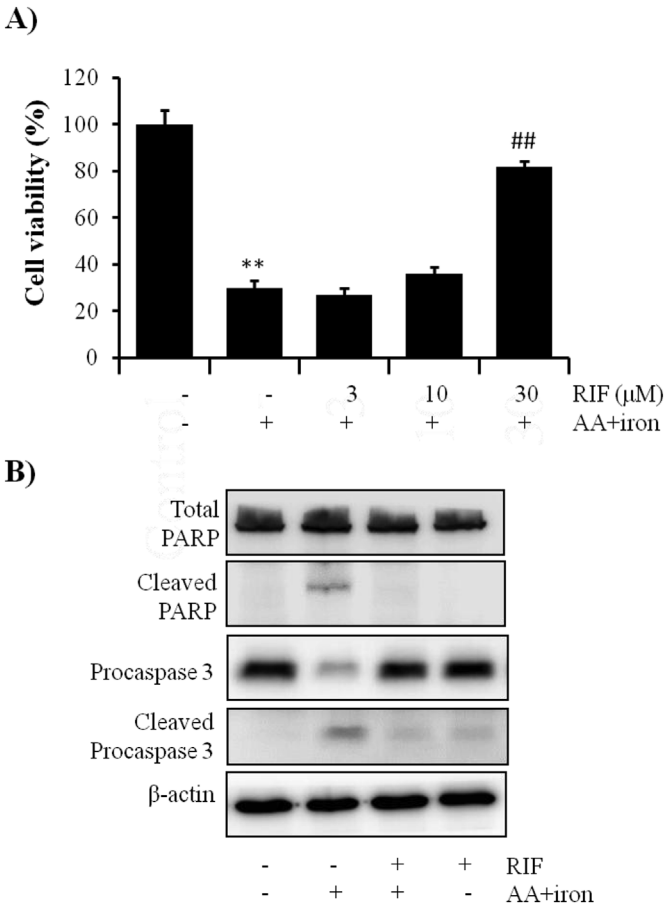


Fig. 2. Effect of rifampicin on oxidative stress in HepG2 cells
(A) Cell viability by MTT assay. HepG2 cells were plated and starved for 12 h prior to rifampicin treatment. One hour after different doses of rifampicin treatment, cells were incubated with 10 μM arachidonic acid (AA) for 12 h and followed by exposure to 5 μM iron for 2 h. (B) Apoptosis related markers expression analyzed by immune blotting analysis. All data represent means ± SD of three independent experiments (**$\rho < 0.01$ between control and AA + iron treated cells; ##$\rho < 0.01$, #$\rho < 0.05$ between AA + iron treated cells with or without rifampicin). AA, arachidonic acid; RIF, rifampicin.

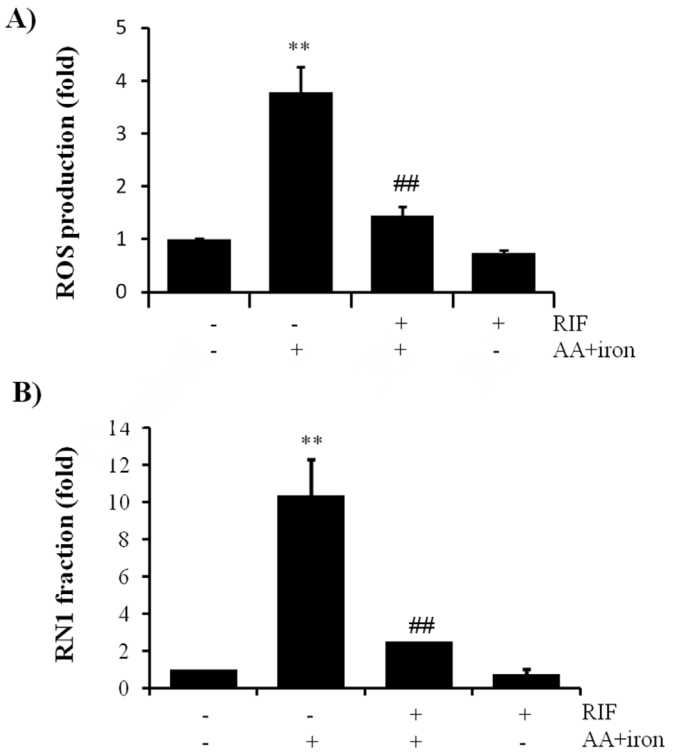


Fig. 3. Effect of rifampicin on mitochondrial membrane potential (MMP)
(A) ROS production measurement by DCFH-DA staining. (B) RN1 fraction (the cell population of the low rhodamine intensity). Mitochondria protective effect of rifampicin was assessed by flow cell analysis. HepG2 cells were treated as describe in Fig. 2A. And then, cells were exposed to 5 μM iron for 2 h followed by 1 h incubation with 10 μM Rh 123. All data represent means ± SD of three independent experiments (**ρ < *0.01* between control and AA + iron treated cells; ##ρ < *0.01*, #ρ < *0.05* between AA + iron treated cells with or without rifampicin). AA, arachidonic acid; RIF, rifampicin.

3.4. Effect of rifampicin on nuclear Nrf2 with AMPKα

Next, we evaluated AMPKα-mediated nuclear localization of Nrf2. HepG2 cells were incubated with 30 μM rifampicin for the indicated time periods. At 1 h of incubation, we observed the highest expression of nuclear Nrf2 (Fig. 5A). In addition to examining Nrf2 expression, we analyzed the expression of HO-1, a target gene of Nrf2 [45]. The HO-1 protein level increased in a time-dependent manner in HepG2 cells (Fig. 5B). To clarify the association between AMPKα and HO-1 expression, we treated cells with compound C. In HepG2 cells, we treated with compound C for 1 h prior to the 30 μM rifampicin treatment. Compound C clearly inhibited production of the phosphorylated (p) form of AMPKα by rifampicin, but AMPKα expression was not affected. Moreover, compound C treatment significantly reversed the expression of HO-1 protein in HepG2 cells (Fig. 5C). Furthermore, in mouse liver tissue, the group treated with 100 mg/kg rifampicin also showed elevated p-ACC and p-AMPKα expressions compared to those in the control group. Moreover, immunohistochemical staining revealed that p-AMPKα was highly expressed in the area adjacent to the central vein of the mouse liver (Fig. 5D). Finally, the rifampicin protected the dysregulation of AMPK phosphorylation by APAP (Fig. 5E).

4. Discussion

Excessive ROS presence induces liver pathogenesis through several steps, including inflammation, fibrosis, cirrhosis, and HCC [9]. Because strong stimuli sometimes induce valuable molecular signals under severe oxidative stress, these cellular toxic environments that we used here (i.e. AA + iron in hepatocyte and APAP in mice) were designed.

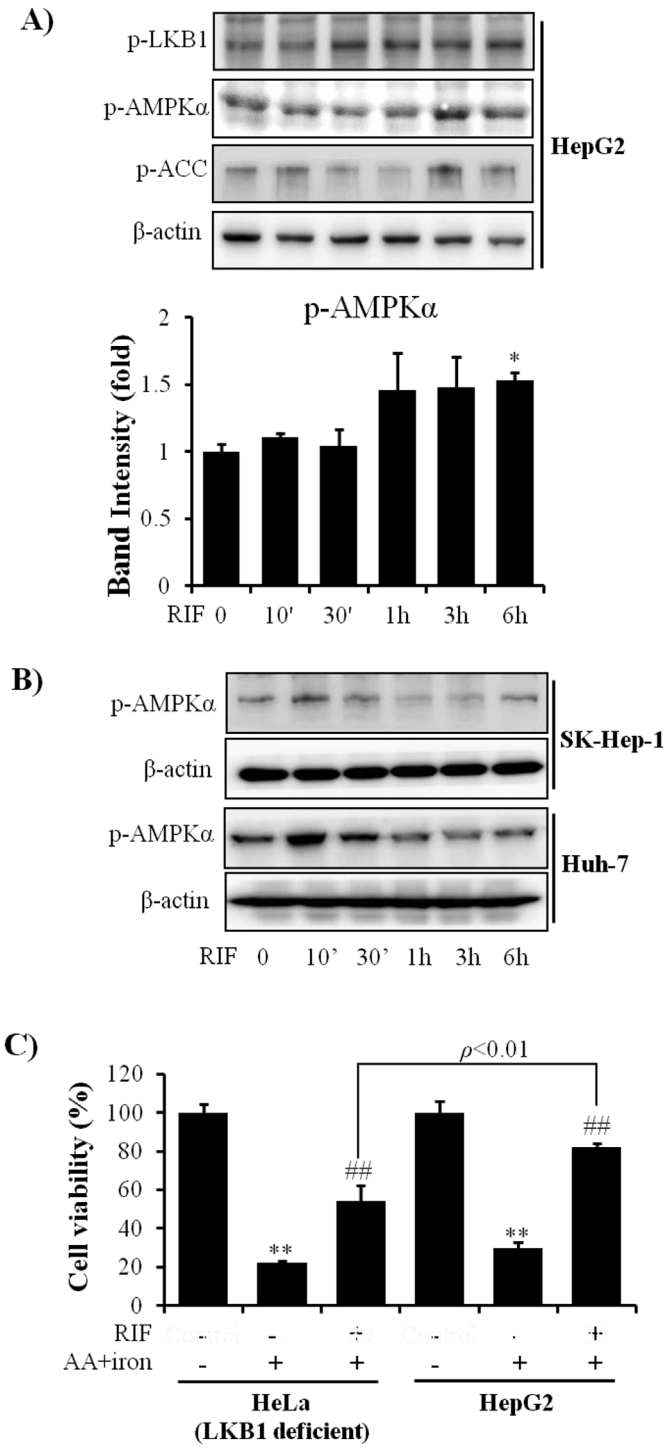


Fig. 4. Effect of rifampicin on AMPKα activation.
Activation of AMPKα in HepG2 (A), SK-Hep-1 and Huh-7 cells (B). HepG2, SK-Hep-1 and Huh-7 cells were plated in 6-well plate. After 12 h starvation, cells were incubated with 30 μM rifampicin for indicated time periods. (C) Comparative cell viability in HeLa cells and HepG2 cells. HeLa cells and HepG2 cells are treated as described in Fig. 2. All data represent means ± SD of three independent experiments (**ρ < *0.01* between control and AA + iron treated cells; ##ρ < *0.01*, #ρ < *0.05* between AA + iron treated cells with or without rifampicin). Data represent the mean ± SD of eight mice. Scale bars = 50 μm. AA, arachidonic acid; RIF, rifampicin.

Here, we investigated the antioxidant effect of rifampicin in hepatocytes. We used the well-established models including AA + iron and APAP as ROS inducers, and determined both the effect of the drug and

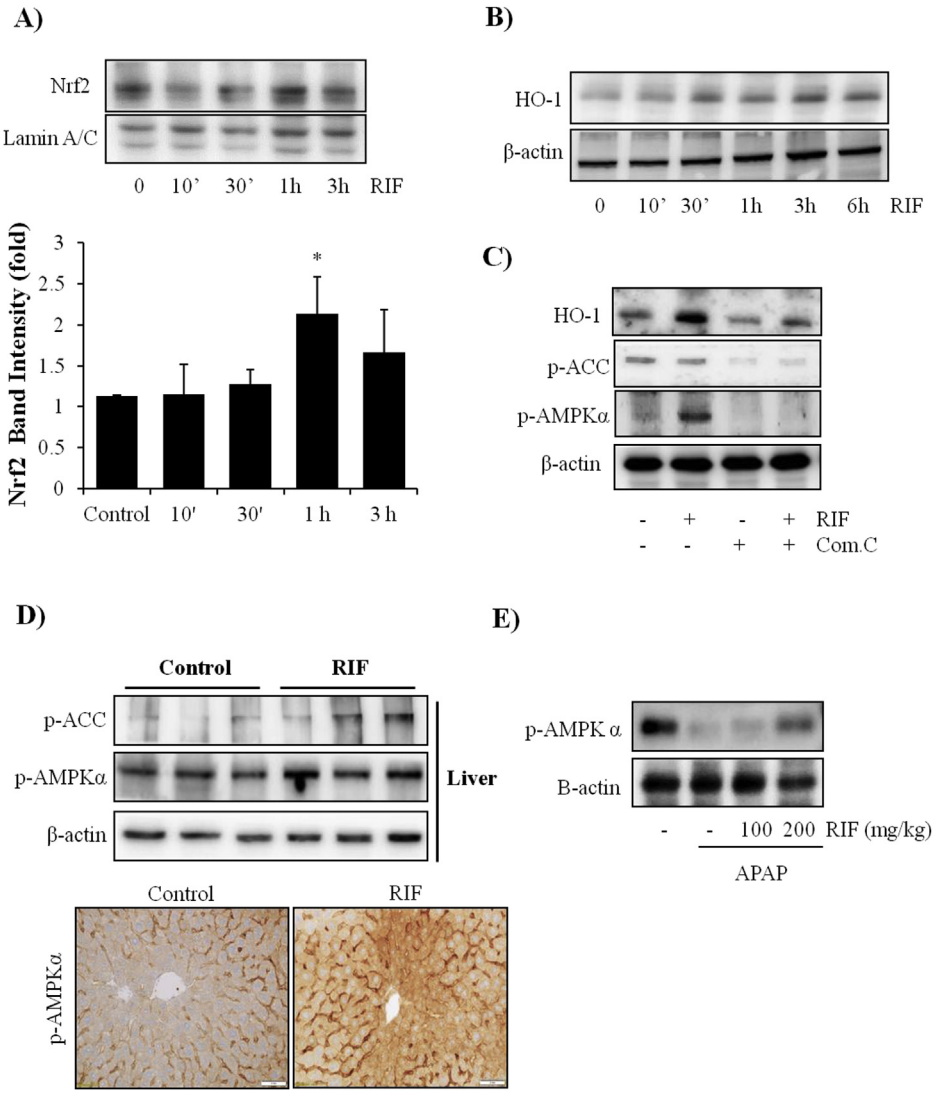


Fig. 5. Effect of rifampicin on Nrf2 signal.
(A) Nuclear Nrf2 expression. Effect of rifampicin on Nrf2 expression was assessed by immunoblot analysis in nuclear extraction of cell stimulated with 30 μM of rifampicin in different time of incubation. Data represent means ± SD of three independent experiments (*ρ < *0.05* between control and cells treated with rifampicin). (B) Effect of rifampicin on HO-1 expression. (C) Effect of compound C on HO-1 induction by rifampicin. HepG2 cells were plated and starved for 12 h and compound C was treated 1 h prior to 30 μM rifampicin treatment for 3 h. (D) Effect of rifampicin on protein expression of p-AMPKα in mouse liver tissues. Mice were orally administrated with 100 mg/kg of rifampicin for three consecutive days. (E) The representative blot of AMPK phosphorylation in the liver tissue. RIF, rifampicin; Com.C, compound C.

the underlying defensive mechanism against oxidative stress.

Under oxidative stress, the released cytochrome *C* binds to Apaf1 and procaspase 9 to form apoptosomes, resulting in the PARP cleavage [46–48]. In our MTT assay results, AA + iron treatment induced low cell viability indicating cell death, while rifampicin treatment prevented cell death. The immunoblotting results showed decreased expression of procaspase-3 and induced cleavage of PARP with rifampicin treatment. Moreover, rifampicin clearly prevented changes in the levels of apoptosis-related proteins. To confirm that ROS presence can cause cell death, we used DCFH-DA, which is easily oxidized by ROS to DCF [49]. The AA + iron group showed significantly strong fluorescence, whereas rifampicin treatment inhibited the oxidation of non-fluorescent DCFH-DA to fluorescent DCF.

In addition, AA + iron treatment induced the loss of mitochondrial membrane potential (MMP) as was detected by flow cytometry with Rh 123 stain; whereas, rifampicin prevented the AA + iron-induced loss of MMP. Rh 123 stains viable mitochondria and is released upon depolarization of the mitochondrial membrane, as indicated by weak fluorescence [50]. Due to the ROS-induced opening of the mPTP, the AA + iron group only showed low-intensity fluorescence. Therefore, we concluded that rifampicin acts as an antioxidant and decided to further investigate the underlying mechanism.

AMPKα has a role as an intracellular sensor of cell energy, and LKB1 is the major upstream kinase of AMPKα and becomes active *via* phosphorylation [38]. LKB1 activates AMPKα by phosphorylating the Thr 172 sites of AMPKα [51]. Activated AMPKα phosphorylates Ser 79 of ACC, turning it to an active form of ACC [52]. Our immunoblotting assays showed that rifampicin-treated HepG2, Sk-Hep-1, and Huh-7 cells exhibited increased expression of p-AMPKα and p-LKB1. Moreover, the MTT assay results for HeLa cells, which are LKB1 deficient cells, showed that rifampicin treatment had partial effects on cell survival. Because the absence of LKB1 does not completely compromise the ability of rifampicin to protect cells from AA + iron toxic effects, rifampicin might activate AMPK via an additional mechanism, which may need to clarify in the future. Based on those results, we assumed that rifampicin can protect hepatocytes by activating the AMPKα pathway; thus, we proceeded to study the mechanism further.

Previous studies have reported that Nrf2 is an antioxidant-related gene transcription factor that is regulated by or cooperates with AMPKα [53–56]. In the absence of cellular stress, Kelch-like ECH-associated protein 1 (Keap1) inhibits nuclear localization of Nrf2 *via* the binding of the double glycine repeat domain with Nrf2 [57]. However, oxidative stress oxidizes the cysteine residue of Keap1 and induces protein modification, leading to detachment of Nrf2 from the Keap1-Nrf2 complex [58,59]. Rifampicin treatment increased the Nrf2 protein expression level in the nucleus and the HO-1 protein level in the cytosol. However, pretreatment of compound C partially blocked the induction of HO-1 by rifampicin. Taken together, these results demonstrate that activated AMPKα translocates cytosolic Nrf2 to the nucleus and transcripts HO-1 protein.

In addition, rifampicin was shown to protect the liver from antioxidant injury, *in vivo*. APAP is metabolized mainly by the liver, and during overdose APAP metabolism, a minor amount of APAP is converted to N-acetyl-p-benzoquinone imine (NAPQI) by CYP2E1 in the liver. NAPQI is a highly reactive toxic metabolite and causes glutathione (GSH) depletion [60,61]. When a drug toxicant is improperly detoxified, ROS diffuse into hepatocytes, which is followed by Kupffer cells and neutrophils inducing inflammation and formation of ROS as a response to the drug's toxicity, leading to mitochondrial dysfunction and oncotic necrosis [62,63].

Both the ALT and AST enzymes serve as markers of liver injury and they leak into the circulatory system during hepatic damage [64]. In this study, rifampicin-treated group showed significantly restrained leakage of ALT and AST to the blood circulatory system. Therefore, we investigated the liver protective effect of rifampicin through both blood and histological analyses. Moreover, rifampicin increased the expressions of p-AMPKα and p-ACC.

Overall, our results show that rifampicin has an inhibitory effect on oxidative stress *in vivo* and *in vitro*. *In vivo*, rifampicin attenuated the oxidative hepatic damage induced by APAP treatment. *In vitro*, rifampicin prevented the oxidative stress-induced cell death, ROS production, mitochondrial damage, and apoptosis. This antioxidant effect was found to involve activation of AMPKα-mediated Nrf2 translocation from cytoplasm to nucleus. Therefore, we propose that the antioxidant effect of rifampicin against AA + iron and APAP treatment is accomplished through the AMPKα and Nrf2 signaling pathways.

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Authorship

E.H.L. and S.Y.B. conducted research, wrote the paper and had primary responsibility for final content. J.Y.P. and Y.W.K. designed research, wrote the paper and had primary responsibility for final content. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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