Rutin Inhibits Oleic Acid Induced Lipid Accumulation *via* Reducing Lipogenesis and Oxidative Stress in Hepatocarcinoma Cells

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Abstract: Excessive lipid accumulation within liver has been proposed to cause obesity, hyperlipidemia, diabetes, and fatty liver disease. Rutin, a common dietary flavonoid that is consumed in fruits, vegetables, and plant-derived beverages, has various biological functions, including antioxidant, anti-inflammatory, and anticancer effects. However, a hypolipidemic effect of rutin on fatty liver disease has not been reported. In this study, we examined the effect of rutin on reducing lipid accumulation in hepatic cells. Hepatocytes were treated with oleic acid (OA) containing with or without rutin to observe the lipid accumulation by Nile red stain. The result showed rutin suppressed OA-induced lipid accumulation and increased adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) activity in hepatocytes. The expression of critical molecule involved in lipid synthesis, sterol regulatory element binding proteins-1 (SREBP-1), was attenuated in rutin-treated cells. Moreover, long-term incubation of rutin inhibited the transcriptions of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGCR), glycerol-3-phosphate acyltransferase (GPAT), fatty acid synthase (FAS), and acetyl-coenzyme carboxylase (ACC). Besides, we also found out the antioxidative effect of rutin by increasing the expression of peroxisome proliferator-activated receptor (PPAR)-α and antioxidative enzymes. Taken together, our findings suggest rutin could attenuate lipid accumulation by decreasing lipogenesis and oxidative stress in hepatocyte.

Keywords: antioxidant enzymes, hepatic lipogenesis, lipid accumulation, rutin

Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation in the absence of significant ethanol consumption. It comprises of a broad spectrum of liver disease ranging from fat accumulation in the liver (hepatic steatosis) to steatohepatitis and latter progressing to cirrhosis in 10% to 25% of cases over 8 to 10 years (Matteoni and others 1999). Liver plays an essential role in lipid metabolism via regulating it lipogenesis and oxidative stress (Madan and others 2006). Several studies suggest that excessive intake of calories, visceral obesity, and insulin resistance will burden liver function and be important risk factors for NAFLD development (Browning and Horton 2004). NAFLD is currently the most common cause of chronic liver disease and an independent risk factor for cardiovascular disease (Targher and others 2007). It is estimated that over 20% of the adult population in developed countries has NAFLD and the incidence in adults and children is rising rapidly because of ongoing epidemics of obesity and type 2 diabetes (Charlton 2004). Therefore, preventing and treating NAFLD are relevant to health promotion.

Dietary fat is one of the most important environmental factors associated with the incidence of NFLD. Recent studies on fatty liver in food science have focused on the searching for functional food ingredients or herbal extracts that can suppress the accumulation of hepatic lipid. Rutin is a common dietary flavonoid that is consumed in fruits, including tomatoes, vegetables, and plant-derived beverages such as tea and wine. It was reported that rutin has several pharmacological properties including antioxidant, anticarcinogenic, cytoprotective, antiplatelet, antithrombic, vasoprotective, and cardioprotective activities (La Casa and others 2000; Sheu and others 2004; Mellou and others 2006; Madan and others 2006). Recent reports have suggested that rutin uptake can significantly decrease the weights of body, liver organ, and adipose tissue as well as the levels of hepatic triglycerides and cholesterol levels in high-fat diet (HFD) rats (Hsu and others 2009). Here, we attempted to examine the hepatic hypolipidemia effect and possible mechanism of rutin on hepatic lipid metabolism.

Materials and Method

Chemicals

Rutin used in this study was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.) and the purity is ≥95% (HPLC). The 3-(4, 5-dimethylthiazol-zyl)-2, 5-diphenylterazolium bromide (MTT), Nile red, oleic acid (OA) and statin were purchased from Sigma-Aldrich. Glutathione peroxidase (GPx), peroxisome proliferator-activated receptor (PPAR)-α, superoxide dismutase (SOD) and sterol regulatory element binding protein (SREBP) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz,

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Calif., U.S.A.). Anti-pThr172-AMPK and anti-AMPK antibodies were purchased from Cell Signaling Technology (Beverly, Mass., U.S.A.). Anti-β-actin and anticatalase antibodies were purchased from Sigma-Aldrich.

Cell culture

Human hepatoma HepG2 cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (HyClone®, Thermo Scientific, Logan, Utah, U.S.A.). The cells were cultured at 37 °C in a humidified atmosphere of 95% air to 5% CO_2.

Cytotoxicity assay

HepG2 cells were seeded at a density of 1×10^5 cells/mL in 24-well plate and incubated with statin, OA, and rutin at various concentrations for 24 h. Thereafter, the medium was changed and 0.5 mg/mL MTT was added to incubate for 4 h. The viable cells were directly proportional to the production of formazan. Following dissolved in isopropanol, the absorbance was read at 563 nm with a spectrophotometer (Hatachi 3210, Hitachi, Tokyo, Japan).

Nile red stain

HepG2 cells were seeded in a 6-well plate (3×10^6 cells/mL) and treated with 600 μM OA and different concentrations of rutin for 24 h. After being washed twice with phosphate buffered saline (PBS), the cells were fixed with 4% formaldehyde for 30 min and then stained with 1 μg/mL Nile red for 30 min at room temperature. After staining, the distribution of lipid in cells was immediately analyzed by FACScan flow cytometer (Becton Dickinson, Mountain View, Calif., U.S.A.). Lipid-bounded Nile red fluorescence was detected using inverted fluorescence microscopy.

Preparation of protein extract of HepG2 cells

The proteins of the cells were harvested in a cold radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 50 mM Tris–base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) containing leupeptin (1.7 μg/mL) and sodium orthovanadate (10 μg/mL). The cell mixture was vortexed at 4 °C for 4 h. All mixtures were then centrifuged at 12000 rpm at 4 °C for 10 min, and the protein contents of the supernatants were determined with the Coomassie Brilliant Blue total protein reagent (Kenlor Industries, Costa Mesa, Calif., U.S.A.) using bovine serum albumin as the standard.

Western blot analysis

Equal amounts of protein samples were subjected to (SDS)-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Millipore, Bedford, Mass., U.S.A.). The membranes were blocked with 5% nonfat milk and then incubated with the 1st antibody at 4 °C overnight. Thereafter, membranes were washed 3 times with 0.1% Tween-20 in PBS and incubated with the secondary antibody conjugated to antimouse horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). The bands were detected and revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed ECL hyperfilm in FUJFILM LAS-3000 (Tokyo, Japan). The protein quantification was determined by densitometry using the FUJFILM-Multi Gauge V2.2 software (FUJFILM, Stockholm, Sweden).

Reverse transcription-polymerase chain reaction (RT-PCR) assay

To determine the mRNA expression level of HMG-CoA reductase (HMGCR), fatty acid synthase (FAS), acetyl-coenzyme carboxylase (ACC), and glycerol-3-phosphate acyltransferase (GPAT) in the HepG2 cells, total RNA was extracted

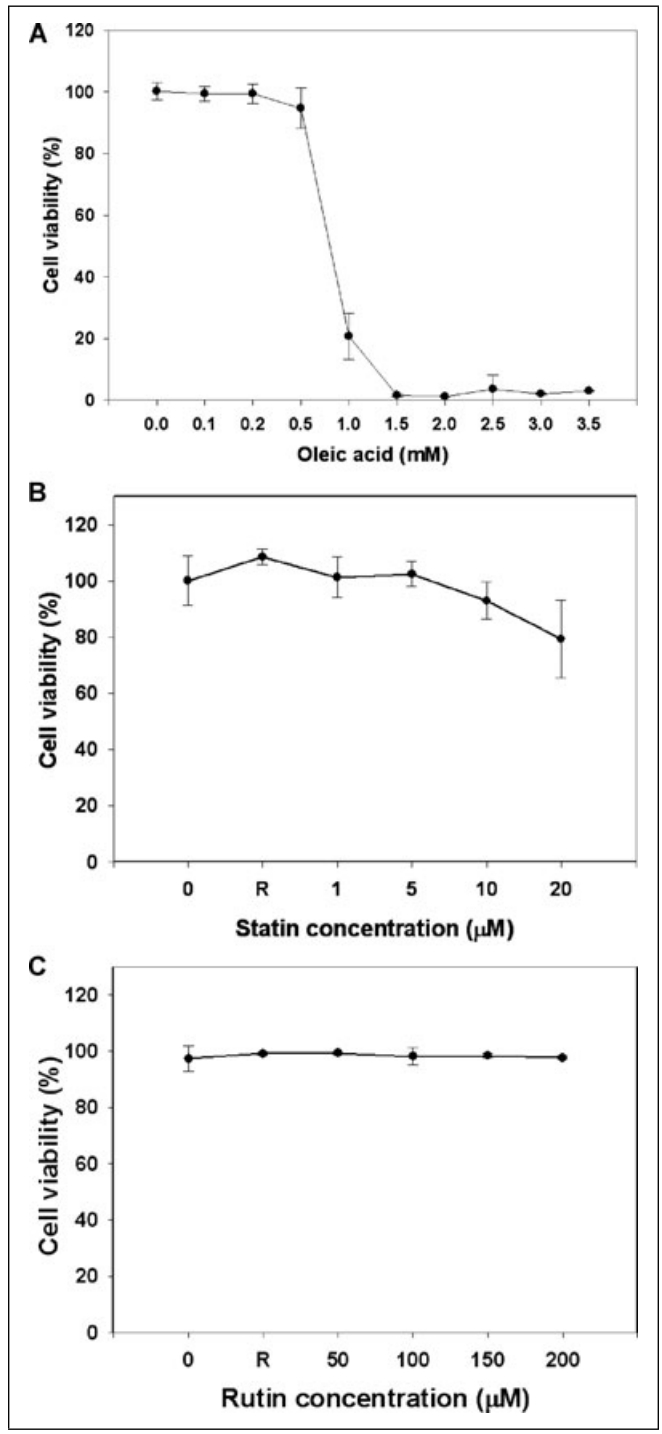


Figure 1–Cytotoxicity of statin, oleic acid, and rutin in HepG2 cells. HepG2 cells were treated with indicated concentrations of OA (A), statin (B), and rutin (C) for 24 h. The viability of HepG2 cells was determined by MTT assay. R: 0.2% DMSO as control. The result was presented as mean ± SD of 3 independent experiments.

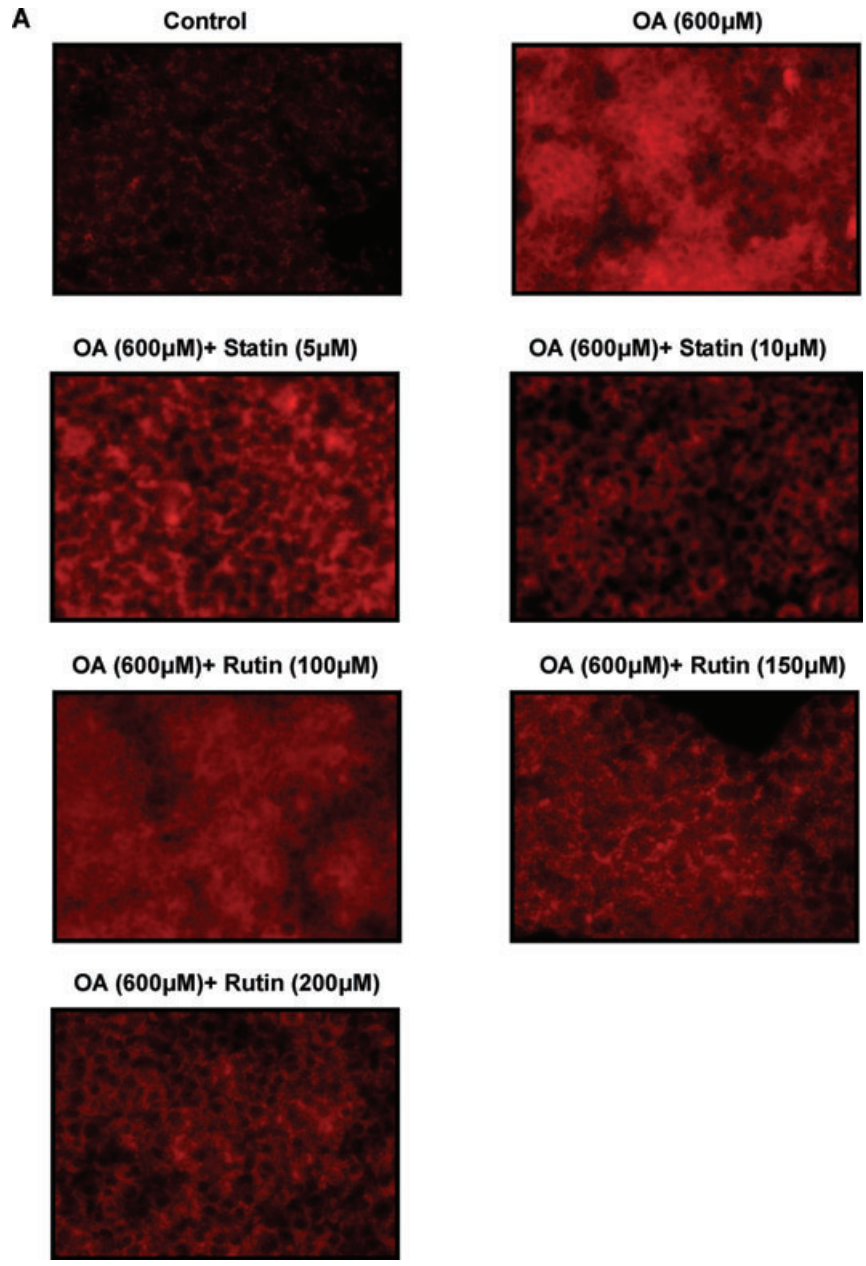
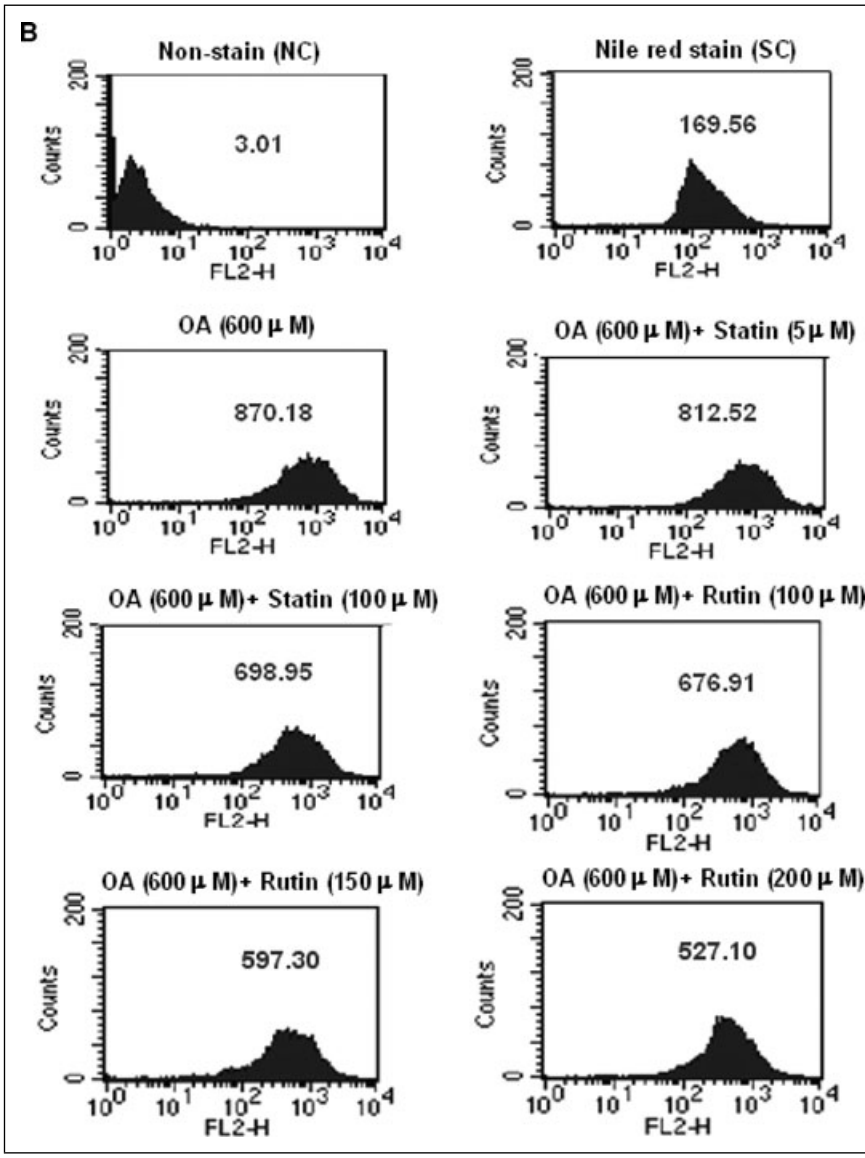


Figure 2–Statin and rutin attenuates oleic acid induced lipid accumulation in HepG2 cells. HepG2 cells were treated with indicated concentrations of statins or rutin in the presence of OA for 24 h. Cells were stained with Nile red (A) and analyzed by flow cytometry (B). Quantitative assessment of the percentage of lipid accumulation. The *x*-axis is Nile red fluorescence intensity and *y*-axis is cell counts. (C) The average of 3 independent experiments. NC = normal control; SC = stain control. The result was expressed as mean ±SD. *, $P < 0.05$. (Continued)

using Trizol Reagent Plus kits (GENEMARK Technology Co., Ltd., Tainan, Taiwan). cDNA synthesis and PCR amplification were performed using the following procedures. For reverse transcription, 4 μg of total cellular RNA were used as templates in a 20 μl reaction containing 4 μl dNTP (2.5 mM), 2.5 μl Oligo dT (10 pmol/μl), and RTase (200 U/μl); the reaction was performed at 42 °C for 1 h. Thereafter, 5 μl cDNA was used as a template for PCR amplification with the appropriate primers. The HMGCR primers were forward: 5′-AGGTTCCAATGGCAACAACAGAAG-3′ and reverse: 5′-ATGCTCCTTGAAC ACCTAGCATCT-3′, which amplified a 828 bp fragment, run for 31 cycles at 95 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min. The FAS primers were forward: 5′- TACATCGACTGCATCAGGCA-3′ and reverse: 5′- GATACTTTCCCGTCGCA TAC-3′, which amplified a 553 bp fragment, run for 40 cycles at 95 °C for 1 min, 58.9 °C for 1 min, and 72 °C for 1 min. The ACC primers were forward: 5′-TGAAGGCTGTGGTGATGGAT-3′ and reverse: 5′-CCGTA GTGGT TGAGG TTGGA-3′, which amplified a 678 bp fragment, run for 40 cycles at 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. The GPAT primers were forward: 5′-ACACCGGTTTCTGACTTTGG-3′ and reverse: 5′-GCCGCTTCTGTTTCTACC AG-3′, which amplified a 589 bp fragment, run for 28 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. The GAPDH primers were forward: 5′-TCCCTCAAGATTGTCAGCAA-3′ and reverse: 5′-AGATCCACAACGGATACA TT-3′, which amplified a 309 bp fragment, run for 30 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The PCR products were visualized on 2% agarose gels stained with ethidium bromide.



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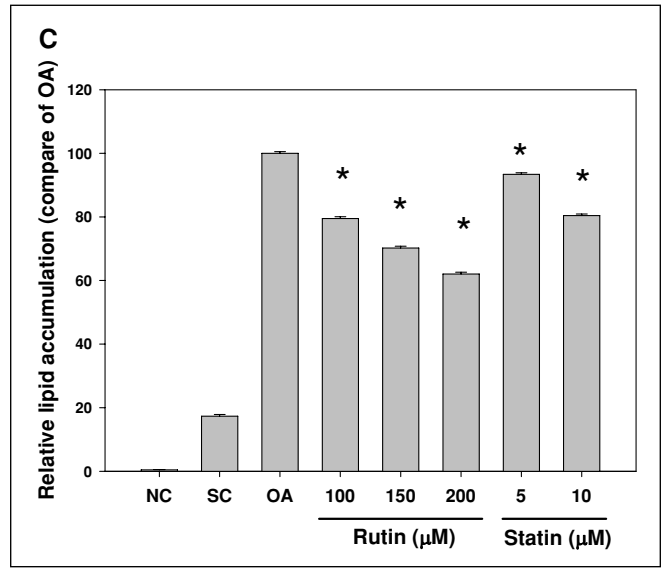
Statistical analysis

Data were analyzed by an unpaired *t*-test and represented as means ± SD. A value of $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Cytotoxicity of statin, oleic acid, and rutin in HepG2 cells

Previous reports using the HepG2 cell line to study lipid metabolism indicated that regulating hepatic LDLR and HMGCR activities could be observed in HepG2 cells (Kong and others 2004; Lu and others 2008). Therefore, the HepG2 cells were used in the present study. We further examined the stain pattern to compare the effect of rutin and statin on lipid homeostasis. Herein, statin (or HMGCR inhibitor), a class of drugs that lowers cholesterol level in human, was used as a positive control. Meanwhile, to avoid cytotoxicity, the viability of cells treated with various concentrations of OA, statin, and rutin was determined by MTT assay. The result indicated the concentration of OA on the inhibition of 50% of $HepG_2$ cells viability (IC_{50}) was 800 μM (Figure 1A). Moreover, there were no cytotoxicity toward $HepG_2$ cells



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below 10 μM statin and 200 μM rutin treatment, respectively (Figure 1B and 1C).

Statin and rutin attenuates lipid accumulation in HepG2 cells

To verify the inhibition of statin and rutin of OA-induced lipid accumulation, HepG2 cells were treated with indicated concentrations of statin and rutin in the presence of OA for 24 h. Then cells were stained with 1 μg/mL Nile red and analyzed by flow cytometry. Both statin and rutin stimulations weakened OA-mediated Nile red stains in a dose-dependent manner (Figure 2A). The quantitative data of Nile red stains displayed 5 and 10 μM statin could reduce 6.63% and 19.68% of lipid accumulation. In response to 100, 150, and 200 μM rutin stimulations, 20.65%, 29.98%, and 38.21% reduction of lipid accumulation were observed (Figure 2B and 2C). Further analyzing the effect of statin and rutin on triglyceride and cholesterol levels showed 5 and 10 μM statin reduced triglyceride level in OA-pretreated cells by 4.2% and 10.06%, respectively. In this regard, treating with 100, 150, and 200 μM of rutin resulted in 3.86%, 3.67%, and 17.85% reduction in triglyceride, respectively (Figure 3A). Additionally, 5 and 10 μM statin stimulation resulted in a reduction of cholesterol by 24.61% and 34.51%, respectively. 150 and 200 μM rutin reduced cholesterol level by 26.5% and 30.5%, respectively. Based on above result, it indicates rutin and statin have the same effect on suppressing OA-mediated lipid accumulation (Figure 2C, 3A, and 3B). Our findings consists with other's reports that confirm rutin could significantly reduce the levels of total cholesterol in animals with a high-cholesterol diet (Ziaee and others 2009) and serum cholesterol and triglyceride levels in rats with streptozotocin-induced diabetes and normal rats (Hardie and Carling 1997; Krishna and others 2005).

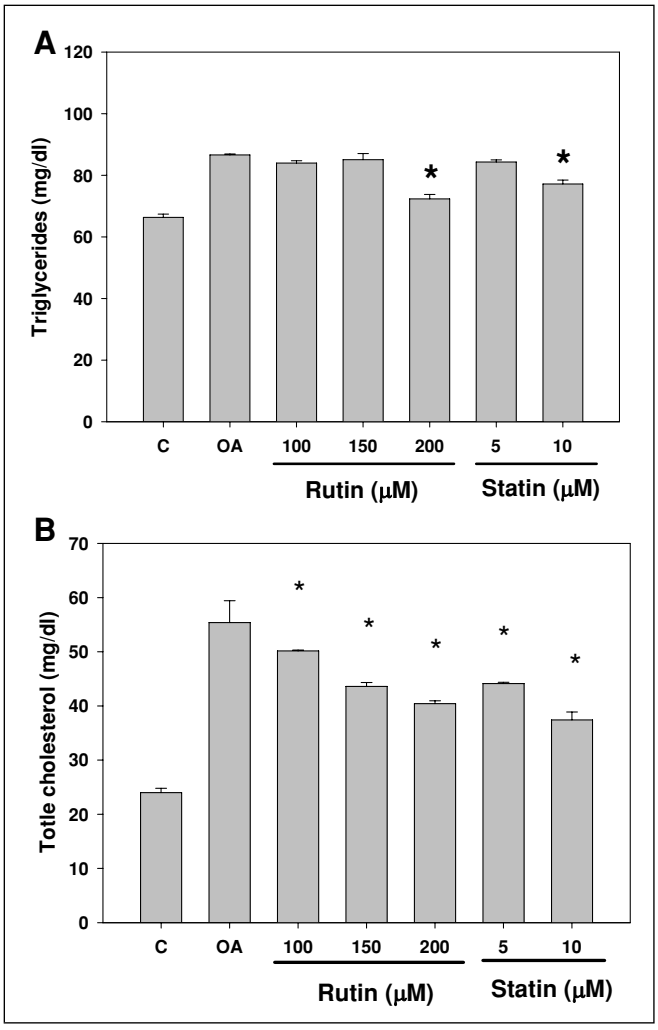

Figure 3–The inhibitory effect of rutin and statin on oleic acid induced triglyceride and cholesterol content in HepG2 cells. Cellular triglyceride (A) and cholesterol (B) were induced by 600 μM OA and cells were treated with indicated concentrations of rutin and statin in the presence of OA for 24 h. Total intracellular triglyceride (A) and cholesterol (B) were analyzed using enzymatic colorimetric method and expressed as mean ± SD = 3, *, $P < 0.05$. OA as control (cells treated with oleic acid only).

Rutin inhibits hepatic lipogenesis in HepG2 cells

The pathogenesis of the fatty change in NAFLD is multifactorial. The mechanism underlying fat accumulation of NAFLD is mostly due to the synthesis of fatty acids and inhibition of fatty-acid oxidation (Reddy and Rao 2006). AMPK is a multisubunit enzyme recognized as a major regulator of lipid biosynthetic pathways due to its role in the phosphorylation and inactivation of key enzymes such as ACC (Zhou and others 2001). Recently, a study suggests AMPK mediates a decrease in SREBP-1 protein expression (Auger and others 2005). SREBP-1 is a key lipogenic transcription factor, which directly activates the expression of more than 30 genes (including FAS), dedicated to the synthesis and uptake of fatty acids, cholesterol, and triglycerides (Brown and Goldstein 1997; Edwards and others 2000; Sakakura and others 2001). We therefore examined the effect of rutin on AMPK activity and the protein expression of A-FABP and SREBP-1. HepG2 cells were pretreated with OA and then exposed to indicated concentrations of rutin for 24 h. Western blot data showed ruitn-treated cells had significant increased phosphorylation level of AMPK up to 1.32-fold ($P < 0.01$). The expression of SREBP-1 was reduced in response to rutin treatment (Figure 4). There are several reports demonstrate AMPK plays a key role in regulating carbohydrate and fat metabolism, serving as a metabolic master switch response to alterations in cellular energy charge (Winder and Hardie 1999). In fact, activation of AMPK has been to validate a strategy for liver steatosis therapy (Brooks and others 2009). Previous studies indicated that polyphenolic extracts from plenty of plants can activate AMPK (Hwang and others 2005) and suppress FAS expression because it prevents SREBP-1 translocation to the nuclei (Auger and others 2005; Weng and others 2007). In this report, we found rutin has the same ability to activate AMPK and then reduce SREBP-1 expression, finally leading to inhibit hepatic lipogenesis.

Rutin promotes hepatic antioxidative ability in HepG2 cells

There are increasing evidences suggesting PPAR-α exerts hypolipidemic effects in the liver through promoting fatty-acid β-oxidation and resulting in decreased fatty acid available for triglyceride synthesis (Yoon 2009). In the impairment or inhibition of PPAR-α function and stimulation of SREBP-1, the receptor molecules controlling the enzymes responsible for the oxidation and synthesis of fatty acids, respectively, appear to contribute to the overall lipid load in the liver (Bugianesi and others 2002; Browning and Horton 2004). In this report, we found out treating $HepG_2$ cells with 200 μM rutin increased PPAR-α expression (Figure 4). Oxidative stress is one of the risk factors linking hyperlipidemia with the pathogenesis of atherosclerosis and nonalcoholic

steatohepatitis (Young and McEneny 2001; Leclercq 2004). It was reported that rutin has several pharmacological properties including antioxidant, anticarcinogenic, and cardioprotective activities (Schwedhelm and others 2003; Mellou and others 2006). Therefore, to reveal the protective effects of rutin on hepatic antioxidant enzymes including catalase, GPx, and SOD, $HepG_2$ cells were exposed to the indicated concentrations of rutin in the presence of OA for 24 h. The result showed that rutin had antioxidative ability by increasing the protein level of catalase, SOD, and GPx by 1.32, 1.21, and 1.42 folds, respectively (Figure 5). Rutin has been identified as the major low-density lipoprotein (LDL) antioxidant compound of mulberry in an *in vitro* study (Ziaee and others 2009). Moreover, it has been reported that rutin could suppress lipid peroxidation in biological membrane systems such as mitochondria, erythrocytes, and others (Middleton and others 2000; Lopez-Revuelta and others 2006). Thus, the other possibility for the hepatoprotective effects of rutin may be related to antioxidant activity to prevent liver injury. However, further studies are needed to clarify the exact mechanism of rutin involved in.

Rutin decreased lipogenesis related gene expression in OA-induced HepG2 cells

Several studies have demonstrated that SREBPs transcriptional regulated the gene for the fatty acid synthesis enzymes (Heemers and others 2001). Another study indicated ACC is the rate-determining step in fatty-acid synthesis (Wakil and others 1983). In fact, previous reports indicated rutin significantly reduced the activity and mRNA levels of various enzymes involved in hepatic fatty-acid synthesis (Odbayar and others 2006). Thus, we used RT-PCR to measure the effect of rutin on the mRNA expressions of FAS, ACC, HMGCR (rate-limiting enzyme for cholesterol synthesis), and GPAT (enzyme for triacylglycerol synthesis). As expected, rutin could clearly decrease the mRNA levels of HMGCR, FAS, ACC, and GPAT in OA-pretreated cells (Fig. 6). Other studies showed fatty acid directly affected some gene expression through regulating transcription factors, including PPAR and SREBP-1 (Clarke 2000). According to the result, we confirmed rutin could decrease lipid synthesis and increase fatty-acid oxidation through activating AMPK-p and PPAR-α, which further

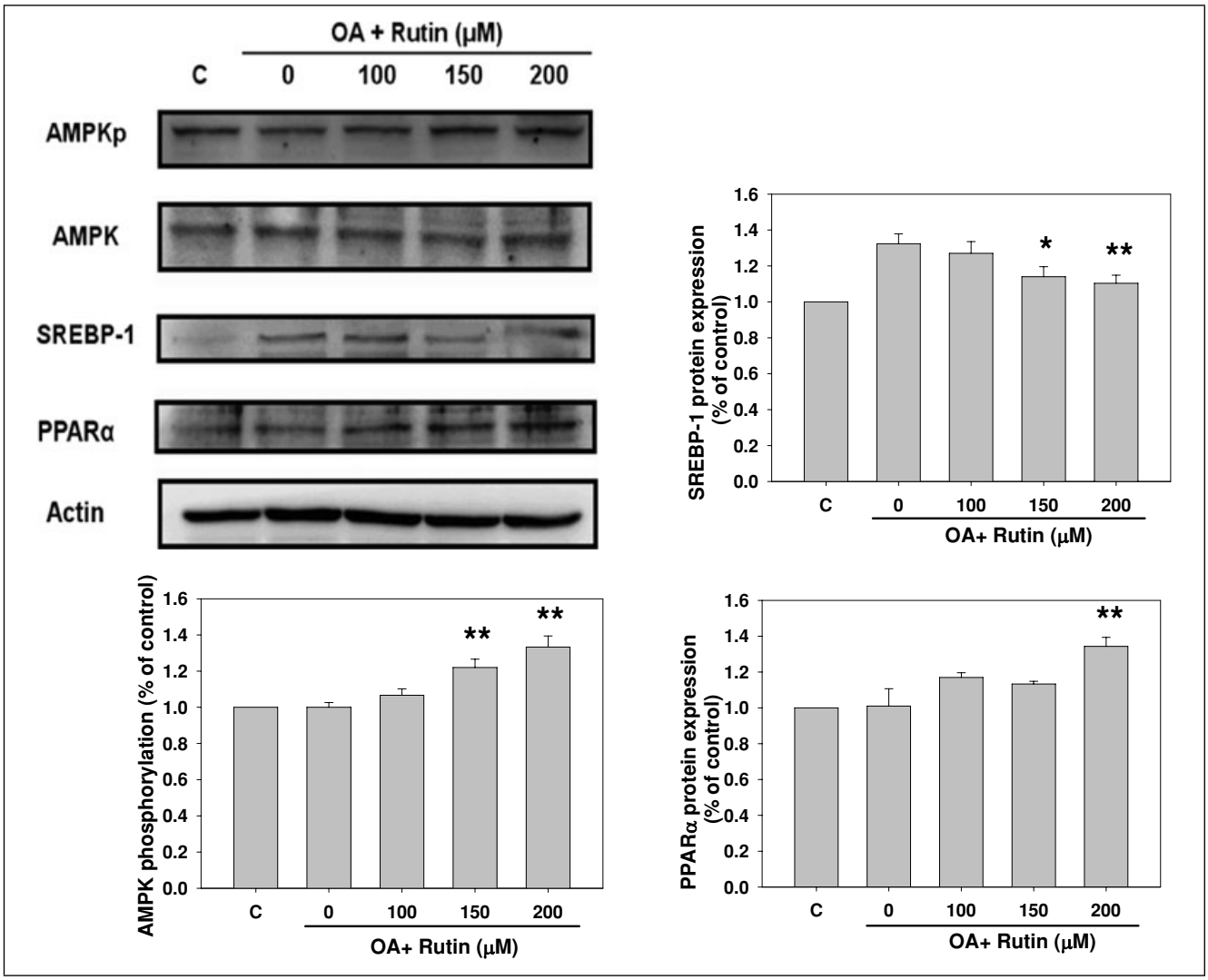

Figure 4–Rutin treatment increases AMPK phosphorylation and PPAR-α level and decreases ACC and SREBP-1 protein expression. $HepG_2$ cells were pretreated 600 μM OA and then incubated with indicated concentrations of rutin for 24 h. pThr172-AMPK, SREBP1, and PPAR-α were detected by Western blot analysis. The numbers below the panels represent quantification of the immunoblot by densitometry. C = control. The result from 3 independent experiments was expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

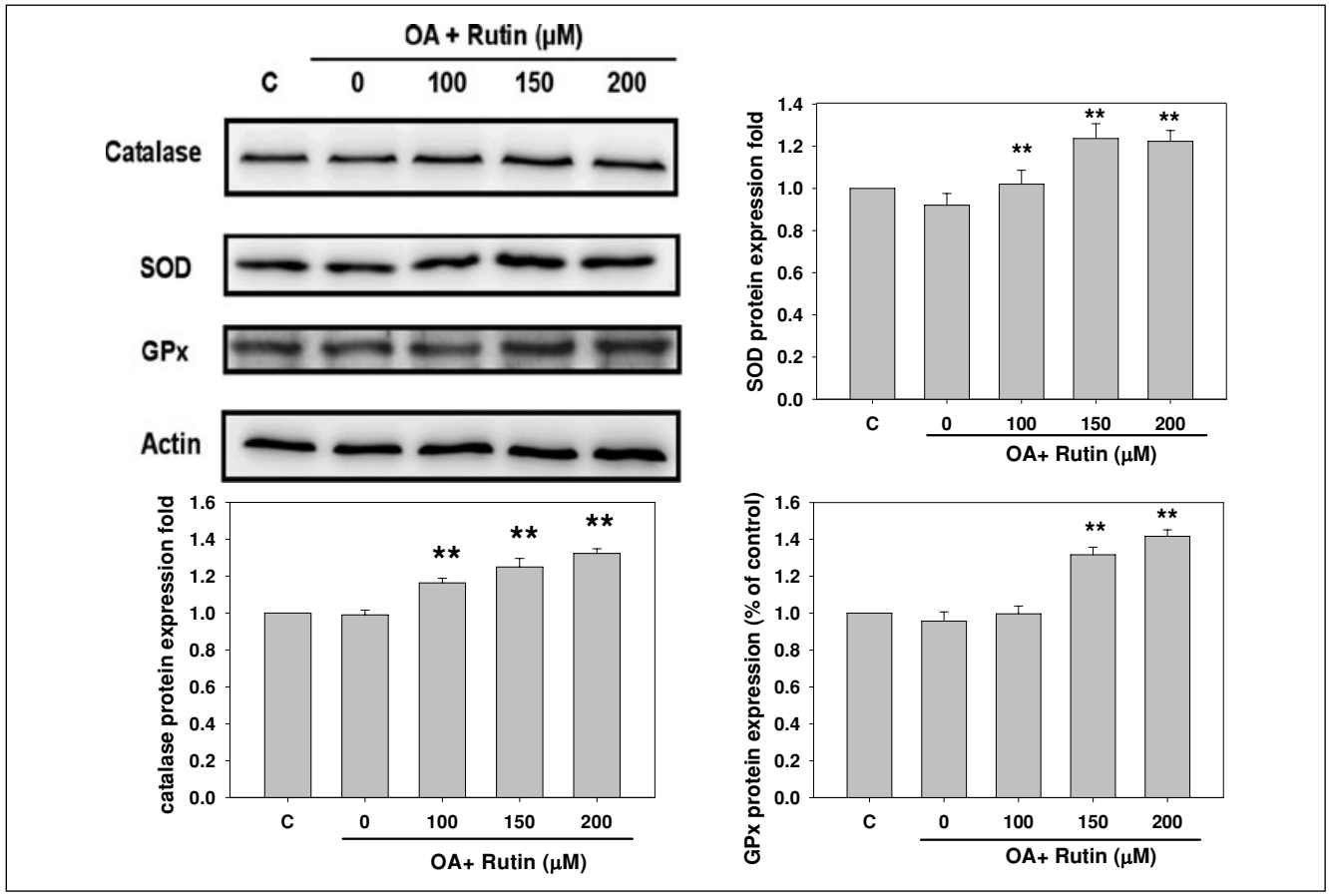

Figure 5–Rutin increases the protein expression of antioxidant enzymes. HepG$_2$ cells were exposed to the indicated concentrations of rutin in the presence of 600 μM OA for 24 h. The catalase, GPx, and SOD-1 protein expressions were detected by Western blot analysis. Data were representative of 3 independent experiments and quantified by densitometric analysis. Expression levels were normalized to β-actin protein level. The results from 3 repeated and separated experiments were similar and expressed as mean ± SD. **, $P < 0.01$.

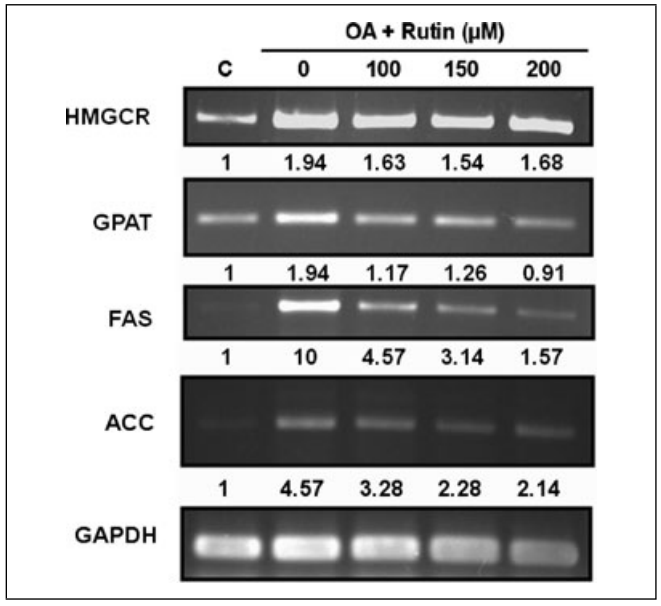

Figure 6–Rutin decreases genes expression related to lipid metabolism in HepG2 cells. The mRNAs of HMGCR, GPAT, FAS, and ACC were extracted from cells treated with the indicated concentrations of rutin in the presence of 600 μM OA for 24 h. The mRNA expression was analyzed by RT-PCR. Expression levels were normalized to GAPDH mRNA expression level. Data were representative of 3 independent experiments and quantified by densitometric analysis.

inhibit protein expression in SREBP-1 and lead to the reduction of the transcription activity of ACC and FAS. Also, rutin could enhance the protein expression of antioxidant enzymes, catalase, SOD, and GPx (Figure 5).

Conclusions

In conclusion, we prove rutin not only reduce lipid accumulation but also had good antioxidant capacity. We also propose AMPK is pivotal in shutting down the anabolic pathway and promoting catabolism by downregulating the activity of key enzymes in lipid metabolism, such as, HMGCR, ACC, and FAS. Rutin suppresses fat accumulation of the liver and could be developed as a potential therapeutic treatment to reduce the formation of a fatty liver.

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