Rutin inhibits oleic acid-induced lipid accumulation via reducing lipogenesis and oxidative stress in hepatocarcinoma cells

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19 **Abstract**

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

38 **Introduction**

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

54 The pathogenesis of the fatty change in NAFLD is multi-factorial. The mechanism
55 underlying fat accumulation of NAFLD is mostly due to the synthesis of fatty acids
56 and inhibition of fatty acid oxidation (Reddy and Rao 2006). Activation of FAS

57 expression through modulation of SREBP-1 has been reported in human breast cancer
58 (Magana and Osborne 1996). Many recent studies have demonstrated that
59 transcriptional regulation of the gene for the enzymes of fatty acid synthesis,
60 including FAS, by SREBPs (Magana and others 2000; Heemers and others 2001). In
61 the impairment or inhibition of PPAR-α function and stimulation of SREBP-1, the
62 receptor molecules controlling the enzymes responsible for the oxidation and
63 synthesis of fatty acids respectively, appear to contribute to the overall lipid load in
64 the liver (Bugianesi and others 2002; Browning and Horton 2004). Further, NAFLD is
65 a risk factor for more serious liver alterations, eventually leading to fibrosis and to
66 non-alcoholic steatohepatitis (NASH) (Raman and Allard 2006). Recently, oxidative
67 stress has been postulated to contribute to NASH development. Several studies
68 indicate depletion of antioxidants such as reduced glutathione (GSH), vitamins C and
69 vitamin E was suggested to occur in NASH patients (Leclercq 2004; Videla and
70 others 2004).
71 Dietary fat is one of the most important environmental factors associated with the
72 incidence of NFLD. Recent studies on fatty liver in food science have focused on the
73 searching for functional food ingredients or herbal extracts that can suppress the
74 accumulation of hepatic lipid. Rutin is a common dietary flavonoid that is consumed
75 in fruits, including tomatoes, vegetables and plant-derived beverages such as tea and

76 wine. It was reported rutin has several pharmacological properties including
77 antioxidant, anticarcinogenic, cytoprotective, antiplatelet, antithrombic,
78 vasoprotective and cardioprotective activities (La Casa and others 2000; Sheu and
79 others 2004; Mellou and others 2006; Melloua and others 2006). Recent reports have
80 suggested rutin uptake can significantly decrease the weights of body, liver organ, and
81 adipose tissue as well as the levels of hepatic triglycerides and cholesterol levels in
82 high-fat diet (HFD) rats (Hsu and others 2009). Here, we attempted to examine the
83 hepatic hypolipidemia effect and possible mechanism of rutin on hepatic lipid
84 metabolism.

85 **Materials and method**

86 **Chemicals**

87 The 3-(4, 5-dimethylthiazol-zyl)-2, 5-diphenylterazolium bromide (MTT), nile red,
88 oleic acid, rutin and statin were purchased from Sigma-Aldrich (St. Louis, Mo.,
89 U.S.A.). GPx, PPARα, SOD and SREBP antibodies were obtained from Santa Cruz
90 Biotechnology (CA, U.S.A.). Anti-pThr172-AMPK and anti-AMPK antibodies were
91 purchased from Cell Signaling Technology (Beverly, MA, U.S.A). Anti-β-actin and
92 anti-catalase antibodies were purchased from Sigma-Aldrich.

93 **Cell culture**

94 Human hepatoma HepG2 cells were obtained from American Type Culture

95 Collection and grown in Dulbecco's modified Eagle's medium supplemented with
96 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM
97 L-glutamine (HyClone®, Thermo scientific). The cells were cultured at 37°C in a
98 humidified atmosphere of 95% air-5% CO_2.

99 **Cytotoxicity assay**

100 HepG2 cells were seeded at a density of 1×10^5 cells/ ml in 24-well plate and
101 incubated with statin, OA and rutin at various concentrations for 24 h. Thereafter, the
102 medium was changed and 0.5 mg/ml MTT was added to incubate for 4 h. The viable
103 cells were directly proportional to the production of formazan. Following dissolved in
104 isopropanol, the absorbance was read at 563 nm with a spectrophotometer (Hatachi
105 3210).

106 **Nile red stain**

107 HepG2 cells were seeded in a 6-well plate (3×10^6 cells /ml) and treated with 600
108 μM OA and different concentrations of rutin for 24 h. After being washed twice with
109 PBS, the cells were fixed with 4% formaldehyde for 30 min and then stained with 1
110 μg/ml nile red for 30 min at room temperature. After staining, the distribution of lipid
111 in cells was immediately analyzed by FACScan flow cytometer (Becton Dickinson).
112 Lipid-bounded nile red fluorescence was detected using inverted fluorescence
113 microscopy.

114 **Preparation of protein extract of HepG2 cells**

115 The proteins of the cells were harvested in a cold RIPA buffer (1% NP-40, 50 mM
116 Tris–base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) containing
117 leupeptin (1.7 μg/ml) and sodium orthovanadate (10 μg/ml). The cell mixture was
118 vortexed at 4°C for 4 h. All mixtures were then centrifuged at 12,000 rpm at 4°C for
119 10 min, and the protein contents of the supernatants were determined with the
120 Coomassie Brilliant Blue total protein reagent (Kenlor Industries, USA) using bovine
121 serum albumin as the standard.

122 **Western blot analysis**

123 Equal amounts of protein samples were subjected to SDS-polyacrylamide gel
124 electrophoresis and electrotransferred to nitrocellulose membranes (Millipore,
125 Bedford, MA, USA). The membranes were blocked with 5% non-fat milk and then
126 incubated with the first antibody at 4°C overnight. Thereafter, membranes were
127 washed three times with 0.1% Tween-20 in PBS and incubated with the secondary
128 antibody conjugated to anti-mouse horseradish peroxidase (GE Healthcare, Little
129 Chalfont, Buckinghamshire, UK). The bands were detected and revealed by enhanced
130 chemiluminescence using ECL western blotting detection reagents and exposed ECL
131 hyperfilm in FUJFILM LAS-3000 (Tokyo, Japan). The protein quantification was
132 determined by densitometry using the FUJFILM-Multi Gauge V2.2 software.

133 **Reverse transcription-polymerase chain reaction (RT-PCR) Assay**

134 To determine the mRNA expression level of HMGCR, FAS, ACC and GPAT in
135 the HepG2 cells, total RNA was extracted using Trizol Reagent Plus kits
136 (GENEMARK Technology Co., Ltd). cDNA synthesis and PCR amplification were
137 performed using the following procedures. For reverse transcription, 4 μg of total
138 cellular RNA were used as templates in a 20 μl reaction containing 4 μl dNTP (2.5
139 mM), 2.5 μl Oligo dT (10 pmol/μl), and RTase (200 U/μl); the reaction was performed
140 at 42°C for 1 h. Thereafter, 5 μl cDNA was used as a template for PCR amplification
141 with the appropriate primers. The HMGCR primers were forward:
142 5′-AGGTTCCAATGGCAACAACAGAAG-3′ and reverse: 5′-ATGCTCCTTGAAC
143 ACCTAGCATCT-3′, which amplified a 828 bp fragment, run for 31 cycles at 95°C for
144 1 min, 63°C for 1 min and 72°C for 1 min. The FAS primers were forward: 5′-
145 TACATCGACTGCATCAGGCA-3′ and reverse: 5′- GATACTTTCCCGTCGCA
146 TAC-3′, which amplified a 553 bp fragment, run for 40 cycles at 95°C for 1 min,
147 58.9°C for 1 min and 72°C for 1 min. The ACC primers were forward:
148 5′-TGAAGGCTGTGGTGATGGAT-3′ and reverse: 5′-CCGTA GTGGT TGAGG
149 TTGGA-3′, which amplified a 678 bp fragment, run for 40 cycles at 95°C for 1 min,
150 52°C for 1 min and 72°C for 1 min. The GPAT primers were forward:
151 5′-ACACCGGTTTCTGACTTTGG-3′ and reverse: 5′-GCCGCTTCTGTTTCTACC

152 AG-3′, which amplified a 589 bp fragment, run for 28 cycles at 95°C for 1 min, 50°C

153 for 1 min and 72°C for 1 min. The GAPDH primers were forward:

154 5′-TCCCTCAAGATTGTCAGCAA-3′ and reverse: 5′-AGATCCACAACGGATACA

155 TT-3′, which amplified a 309 bp fragment, run for 30 cycles at 95°C for 1 min, 55°C

156 for 1 min and 72°C for 2 min. The PCR products were visualized on 2% agarose gels

157 stained with ethidium bromide.

158 **Statistical analysis**

159 Data was analyzed by an unpaired *t*-test and represented as means ± SD. A value

160 of $p < 0.05$ was considered statistically significant.

161 **Results and discussion**

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

163 Previous reports using the HepG2 cell line to study lipid metabolism indicated that

164 regulating hepatic LDLR and HMGCR activities could be observed in HepG2 cells

165 (Kong and others 2004; Lu and others 2008). Therefore, the HepG2 cells were used in

166 the present study. We further examined the stain pattern to compare the effect of rutin

167 and statin on lipid homeostasis. Herein, statin (or HMGCR inhibitor), a class of drugs

168 that lowers cholesterol level in human, was used as a positive control. Meanwhile, to

169 avoid cytotoxicity, the viability of cells treated with various concentrations of OA,

170 statin and rutin was determined by MTT assay. The result indicated the concentration

171 of OA on the inhibition of 50 % of $HepG_2$ cells viability (IC_{50}) was 800 μM (Figure

172 1A). Moreover, there were no cytotoxicity toward $HepG_2$ cells below 10 μM statin

173 and 200 μM rutin treatment, respectively (Figure 1B and 1C).

174 **Statin and rutin attenuates lipid accumulation in HepG2 cells**

175 To verify the inhibition of statin and rutin of OA-induced lipid accumulation,

176 HepG2 cells were treated with indicated concentrations of statin and rutin in the

177 presence of OA for 24 h. Then cells were stained with 1μg/ml nile red and analyzed

178 by flow cytometry. Both statin and rutin stimulations weakened OA-mediated nile red

179 stains in a dose-dependent manner (Figure 2A).The quantitative data of nile red stains

180 displayed 5 μM and 10 μM statin could reduce 6.63% and 19.68% of lipid

181 accumulation. In response to 100 μM, 150 μM and 200 μM rutin stimulations, 20.65%,

182 29.98% and 38.21% reduction of lipid accumulation were observed (Fig. 2B and 2C).

183 Further analyzing the effect of statin and rutin on triglyceride and cholesterol levels

184 showed 5 μM and 10 μM statin reduced triglyceride level in OA-pretreated cells by

185 4.2% and 10.06% respectively. In this regard, treating with 100 μM, 150 μM and 200

186 μM of rutin resulted in 3.86%, 3.67% and 17.85% reduction in triglyceride,

187 respectively (Figure 3A). Additionally, 5 μM and 10 μM statin stimulation resulted in

188 a reduction of cholesterol by 24.61% and 34.51%, respectively. 150 μM and 200 μM

189 rutin reduced cholesterol level by 26.5% and 30.5%, respectively. Based on above

190 result, it indicates rutin and statin have the same effect on suppressing OA-mediated
191 lipid accumulation (Fig. 2C、3A and 3B). Our findings consists with other's reports
192 that confirm rutin could significantly reduce the levels of total cholesterol in animals
193 with a high-cholesterol diet (Ziaee and others 2009) and serum cholesterol and
194 triglyceride levels in rats with streptozotocin-induced diabetes and normal rats
195 (Hardie and Carling 1997; Krishna and others 2005).

196 **Rutin inhibits hepatic lipogenesis in HepG2 cells**

197 AMPK is a multisubunit enzyme recognized as a major regulator of lipid
198 biosynthetic pathways due to its role in the phosphorylation and inactivation of key
199 enzymes such as ACC (Zhou and others 2001). Recently, a study suggests AMPK
200 mediates a decrease in SREBP-1 protein expression (Auger and others 2005).
201 SREBP-1 is a key lipogenic transcription factor which directly activates the
202 expression of more than 30 genes dedicated to the synthesis and uptake of fatty acids,
203 cholesterol and triglycerides (Brown and Goldstein 1997; Edwards and others 2000;
204 Sakakura and others 2001). We therefore examined the effect of rutin on AMPK
205 activity and the protein expression of A-FABP and SREBP-1. HepG2 cells were
206 pre-treated with OA and then exposed to indicated concentrations of rutin for 24 h.
207 Western blot data showed ruitn-treated cells had significant increased
208 phosphorylation level of AMPK up to 1.32 fold (p<0.01). The expression of SREBP-1

209 was reduced in response to rutin treatment (Fig. 4). There are several reports
210 demonstrate AMPK plays a key role in regulating carbohydrate and fat metabolism,
211 serving as a metabolic master switch response to alterations in cellular energy charge
212 (Winder and Hardie 1999). In fact, activation of AMPK has been to validate a strategy
213 for liver steatosis therapy (Brooks and others 2009). Previous researches indicated
214 that polyphenolic extracts from plenty of plants can activate AMPK (Hwang and
215 others 2005) and suppress FAS expression because it prevents SREBP-1 translocation
216 to the nuclei (Auger and others 2005; Weng and others 2007). In this report, we found
217 rutin has the same ability to activate AMPK and then reduce SREBP-1 expression,
218 finally leading to inhibit hepatic lipogenesis.

219 **Rutin promotes hepatic antioxiadtive ability in HepG2 cells**

220 There are increasing evidences suggesting PPAR-α exerts hypolipidemic effects in
221 the liver through promoting fatty acid β-oxidation and resulting in decreased fatty acid
222 available for triglyceride synthesis (Yoon 2009). In this report, we found out treating
223 $HepG_2$ cells with 200μM rutin increased PPAR-α expression (Fig. 4). Oxidative stress
224 is one of the risk factors linking hyperlipidemia with the pathogenesis of
225 atherosclerosis and non-alcoholic steatohepatitis (Young and McEneny 2001;
226 Leclercq 2004). It was reported rutin has several pharmacological properties including
227 antioxidant, anticarcinogenic, and cardioprotective activities (Schwedhelm and others

228 2003; Mellou and others 2006). Therefore, to reveal the protective effects of rutin on
229 hepatic antioxidant enzymes including catalase, glutathione peroxidase (GPx) and
230 superoxide dismutase (SOD), $HepG_2$ cells were exposed to the indicated
231 concentrations of rutin in the presence of OA for 24 h. The result showed rutin had
232 antioxidative ability by increasing the protein level of catalase, SOD and GPx by 1.32,
233 1.21 and 1.42 folds, respectively (Figure 5). Rutin has been identified as the major
234 low-density lipoprotein (LDL) antioxidant compound of mulberry in an *in vitro* study
235 (Ziaee and others 2009). Moreover, it has been reported rutin could suppress lipid
236 peroxidation in biological membrane systems such as mitochondria, erythrocytes, and
237 others (Middleton and others 2000; Lopez-Revuelta and others 2006). Thus, the other
238 possibility for the hepatoprotective effects of rutin may be related to antioxidant
239 activity to prevent liver injury. However, further studies are needed to clarify the
240 exact mechanism of rutin involved in.

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

242 Several studies have demonstrated that SREBPs transcriptional regulated the gene
243 for the fatty acid synthesis enzymes (Heemers and others 2001). Another study
244 indicated ACC is the rate-determining step in fatty acid synthesis (Wakil and others
245 1983). In fact, previous reports indicated rutin significantly reduced the activity and
246 mRNA levels of various enzymes involved in hepatic fatty acid synthesis (Odbayar

247 and others 2006). Thus, we used RT-PCR to measure the effect of rutin on the mRNA
248 expressions of FAS, ACC, HMGCR (rate-limiting enzyme for cholesterol synthesis)
249 and GPAT (enzyme for triacylglycerol synthesis). As expected, rutin could clearly
250 decrease the mRNA levels of HMGCR, FAS, ACC and GPAT in OA-pretreated cells.
251 Other studies showed fatty acid directly affected some gene expression through
252 regulating transcription factors, including PPAR and SREBP-1 (Clarke 2000).
253 According to the result, we confirmed rutin could decrease lipid synthesis and
254 increase fatty acid oxidation through activating AMPK-p and PPAR-α, which further
255 inhibit protein expression in SREBP-1 and lead to the reduction of the transcription
256 activity of ACC and FAS. Also, rutin could enhance the protein expression of
257 antioxidant enzymes, catalase, SOD and GPx (Figure 5).

258 **Conclusions**

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

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401
402
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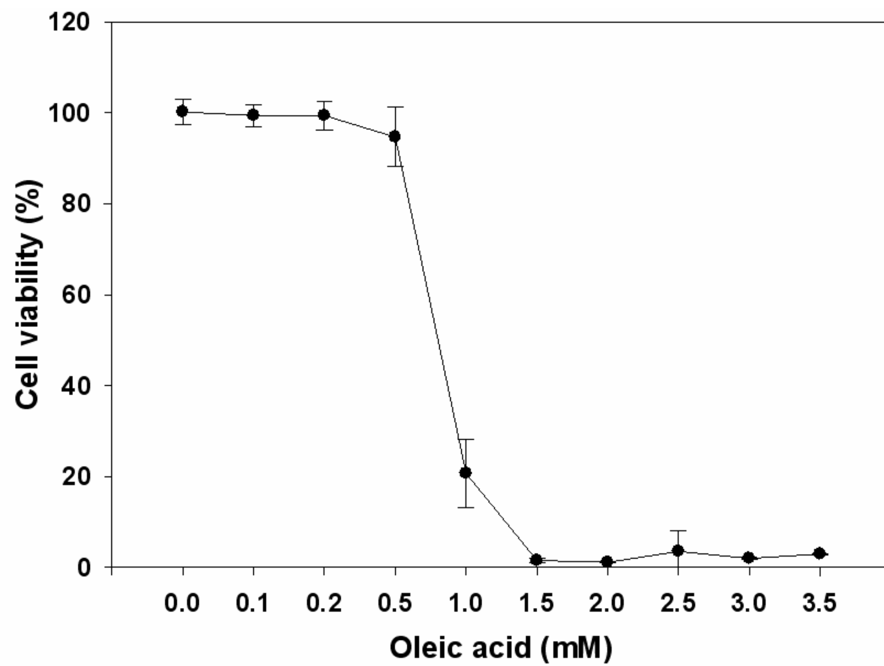
406

407 **Figure 1**

408 **(A)**



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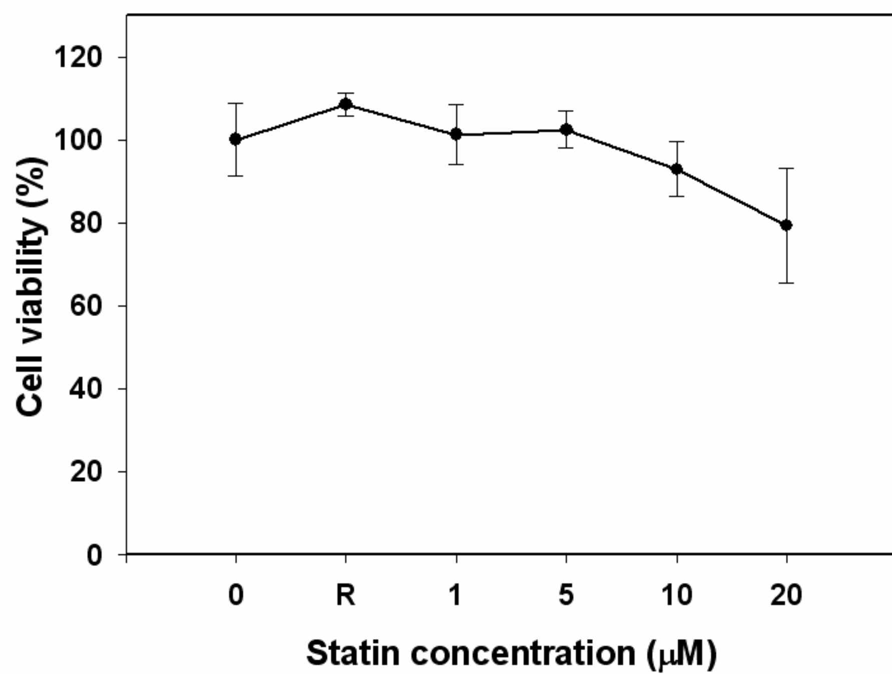
416 **(B)**



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(C)

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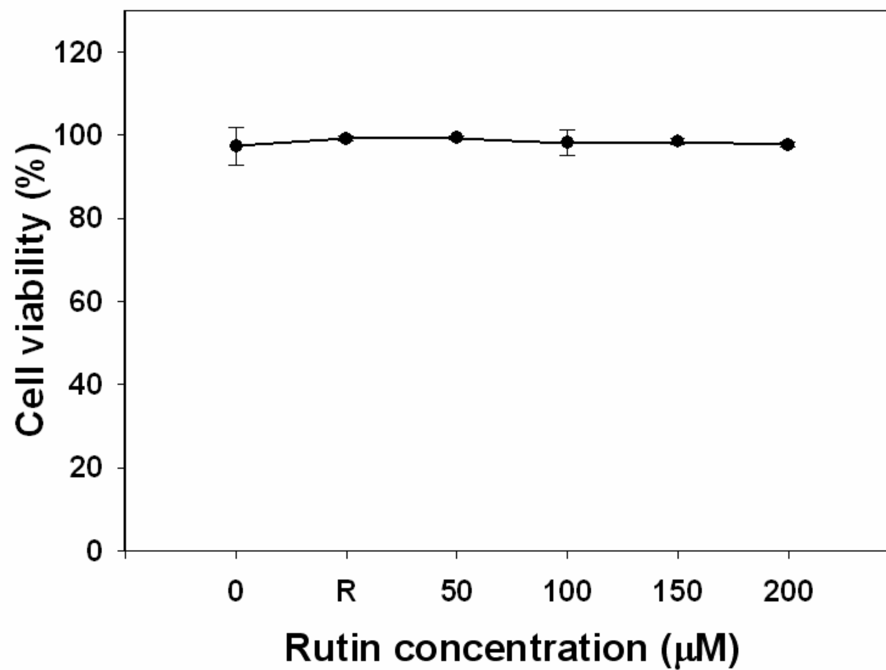

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435 **Figure 1 Cytotoxicity of statin, oleic acid and rutin in HepG2 cells.** HepG2 cells

436 were treated with indicated concentrations of OA (A)、statin (B) and rutin (C) for 24 h.

437 The viability of HepG2 cells was determined by MTT assay. R: 0.2% DMSO as

438 control. The result was presented as mean ± SD of three independent experiments.

439 **Figure 2**

440 (A)

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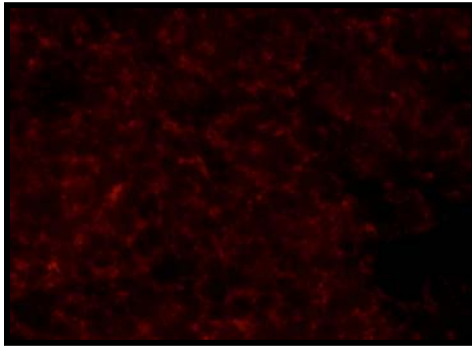
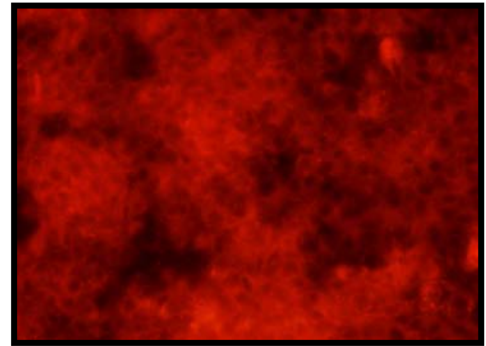
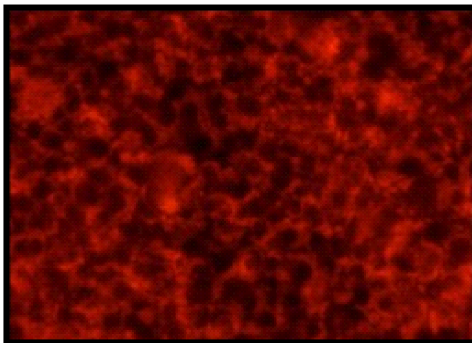


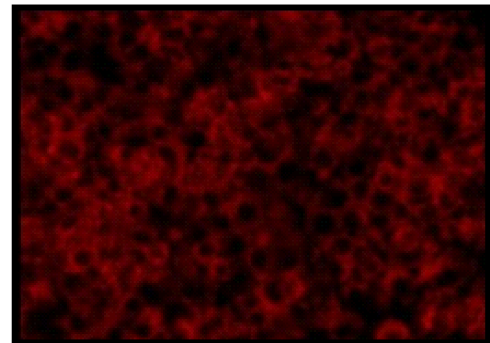
OA (600μM)



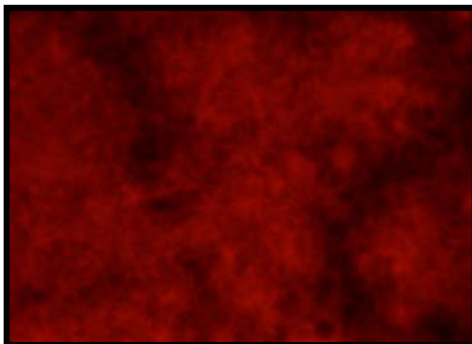
OA (600μM)+ Statin (5μM)



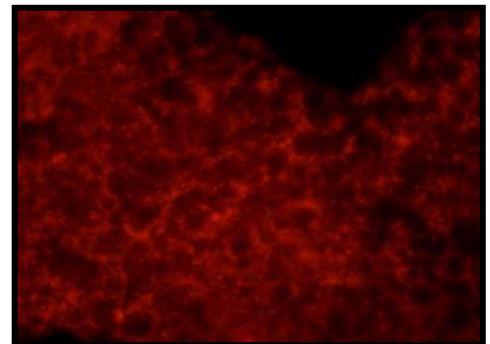
OA (600μM)+ Statin (10μM)



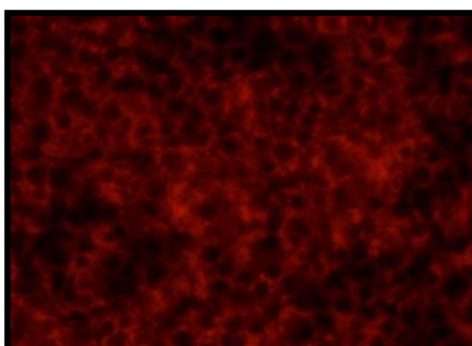
OA (600μM)+ Rutin (100μM)



OA (600μM)+ Rutin (150μM)



OA (600μM)+ Rutin (200μM)



458 **(B)**

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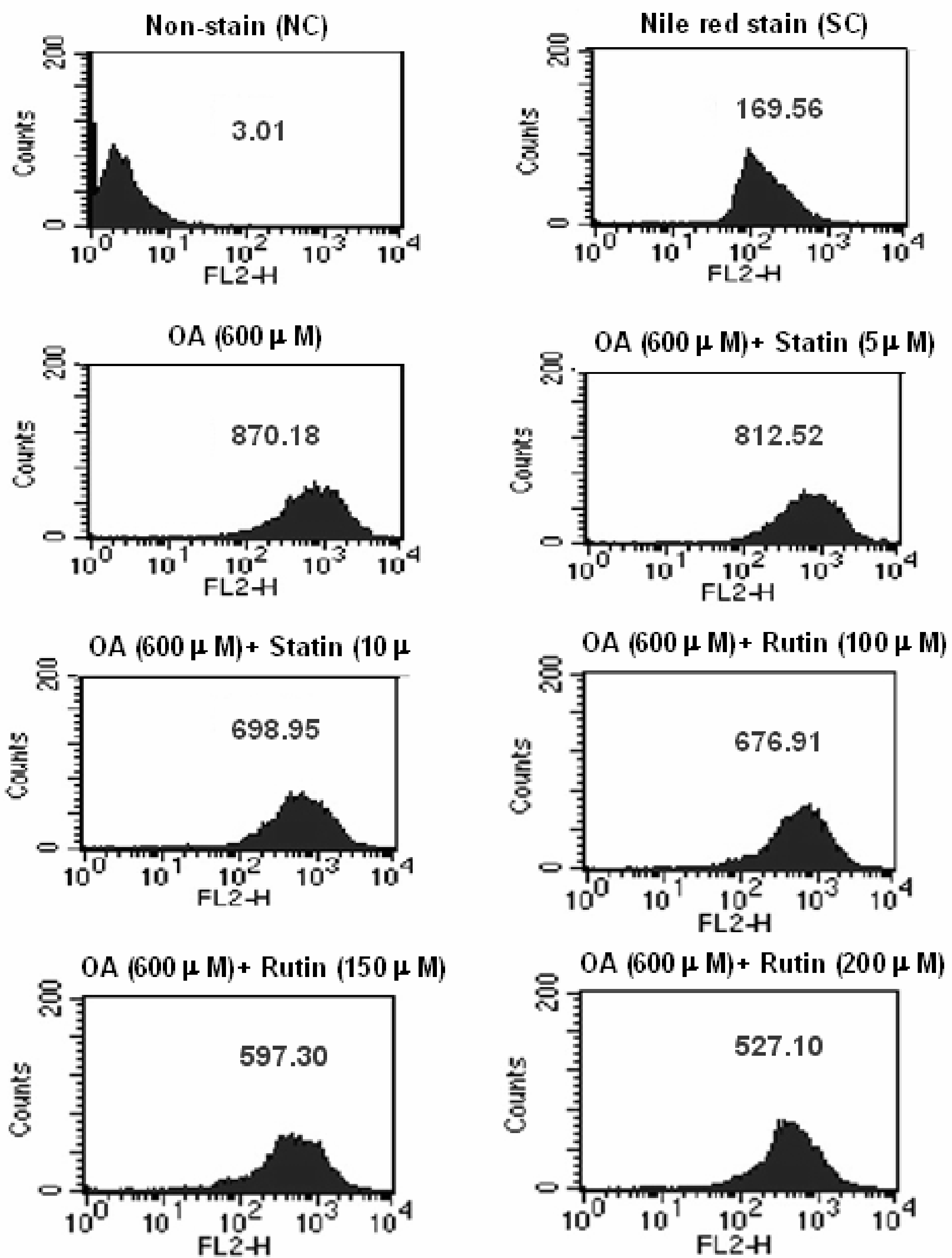

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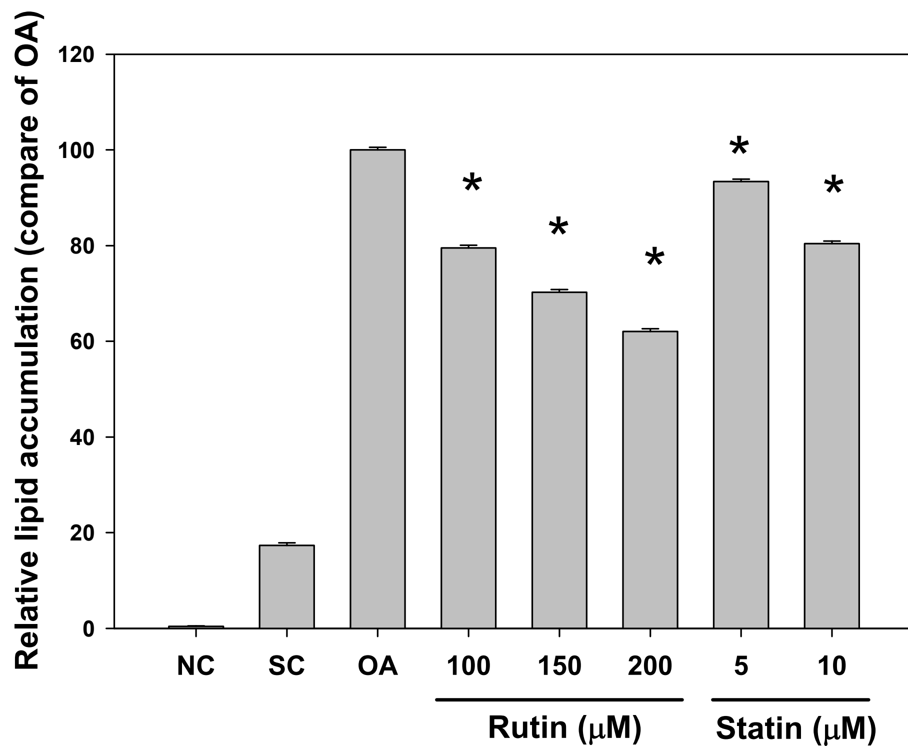

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488 **Figure 2 Statin and rutin attenuates oleic acid-induced lipid accumulation in**

489 **HepG2 cells.** HepG2 cells were treated with indicated concentrations of statinc or

490 rutin in the presence of OA for 24 h. Cells were stained with nile red (A) and analyzed

491 by flow cytometry (B). Quantitative assessment of the percentage of lipid

492 accumulation (C) represented the average of three independent experiments. NC:

493 normal control; SC: stain control. The result was expressed as mean ±SD. *, $p<0.05$.

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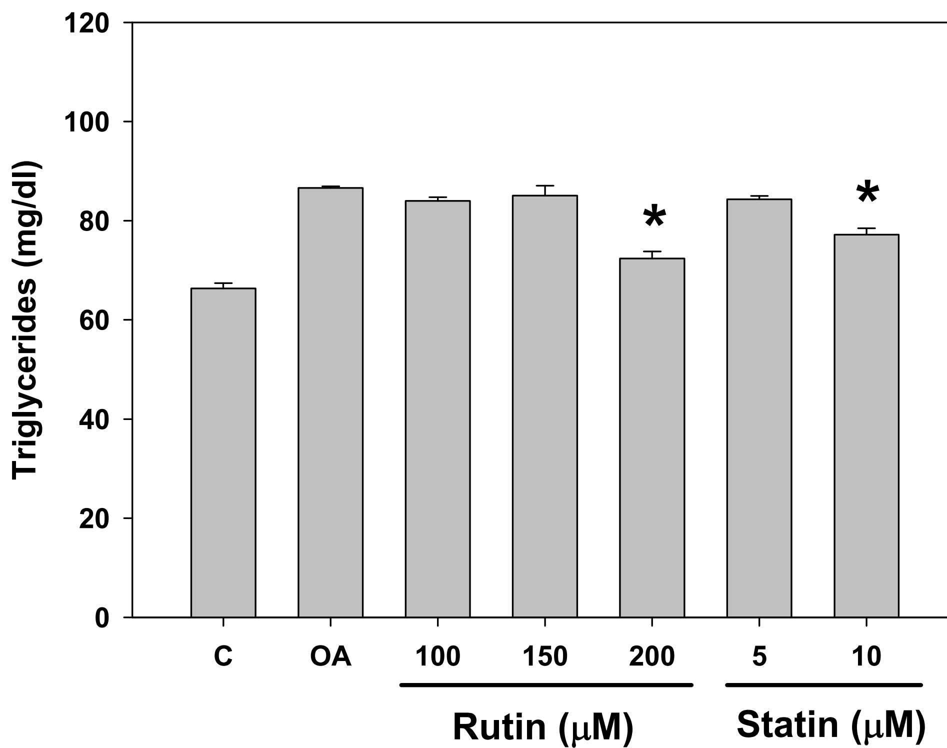
495 **Figure 3.**

496 (A)



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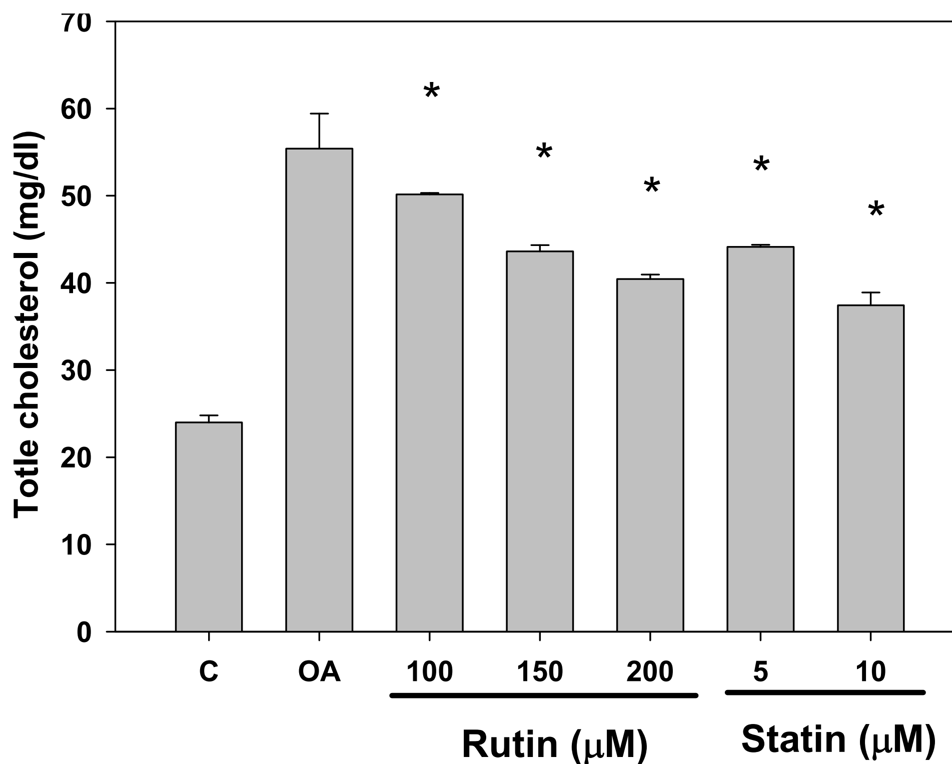
505 (B)



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514 **Figure 3 The inhibitory effect of rutin and statin on oleic acid-induced**
515 **triglyceride and cholesterol content in HepG2 cells.** Cellular triglyceride (A) and
516 cholesterol (B) were induced by 600 μM OA and. Cells were treated with indicated
517 concentrations of rutin and statin in the presence of OA for 24 h. Total intracellular
518 triglyceride (A) and cholesterol (B) were analyzed using enzymatic colorimetric
519 method and expressed as mean ± SD, n=3, *, $p<0.05$. OA as control (cells treated with
520 oleic acid only).
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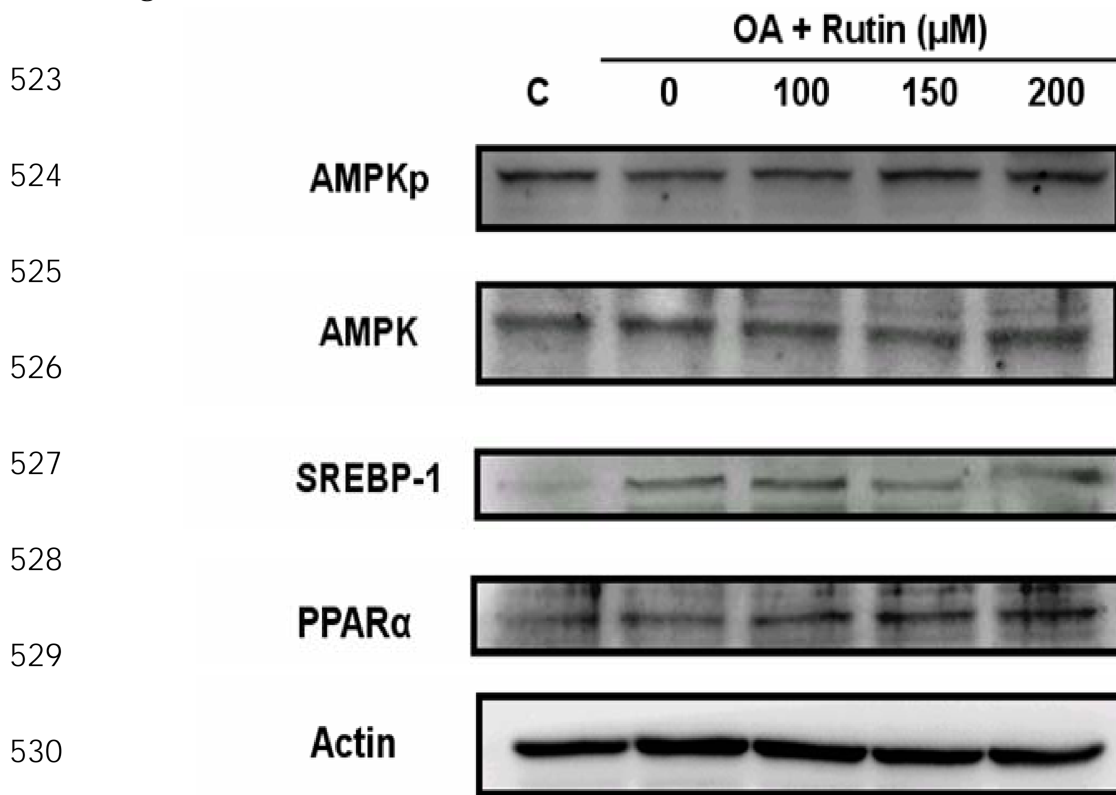
522 **Figure 4.**



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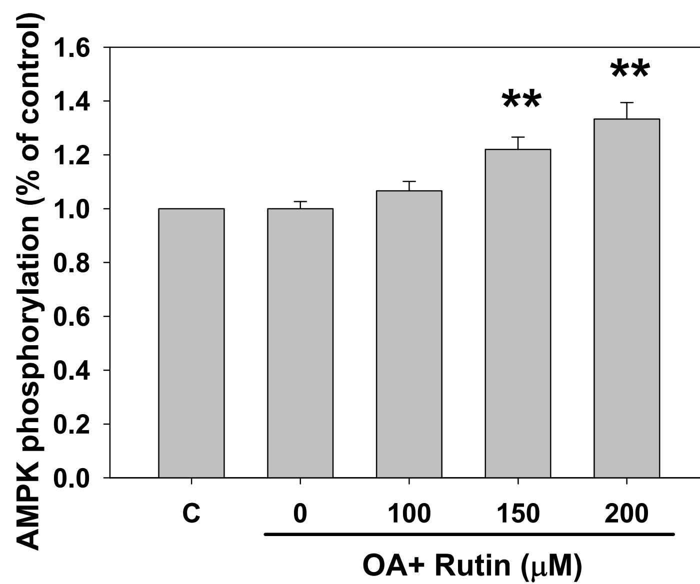

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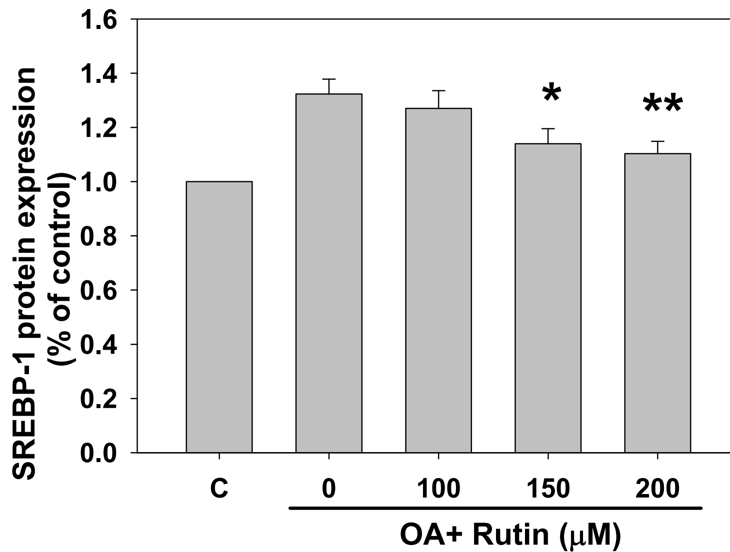

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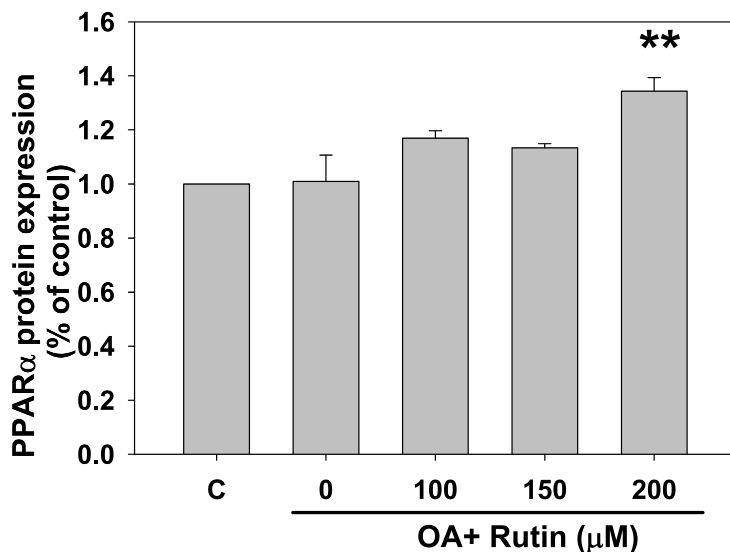

551 **Figure 4. Rutin treatment increases AMPK phosphorylation and PPARα level**
552 **and decreases ACC and SREBP-1 protein expression.** $HepG_2$ cells were pre-treated
553 600 μM OA and then incubated with indicated concentrations of rutin for 24 h.
554 pThr172-AMPK, SREBP1 and PPAR-α were detected by Western blot analysis. The
555 numbers below the panels represent quantification of the immunoblot by densitometry.
556 C: control. The result from three independent experiments was expressed as mean
557 ±SD. *, $p<0.05$; **, $p<0.01$.

558 **Figure 5.**

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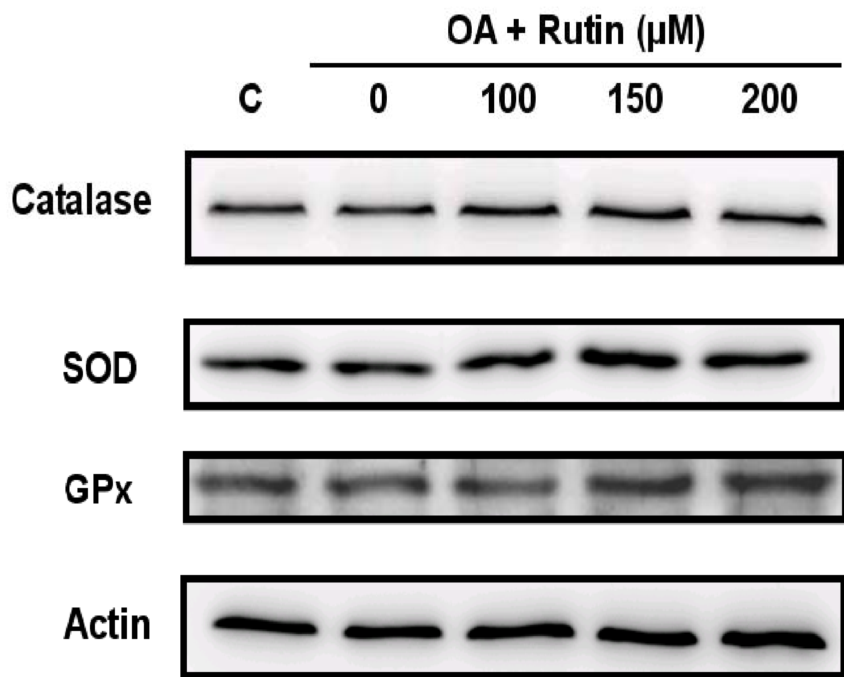

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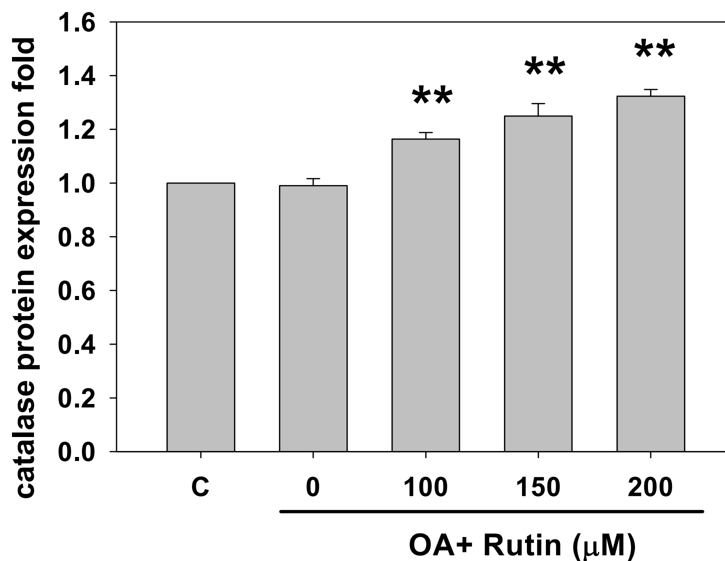

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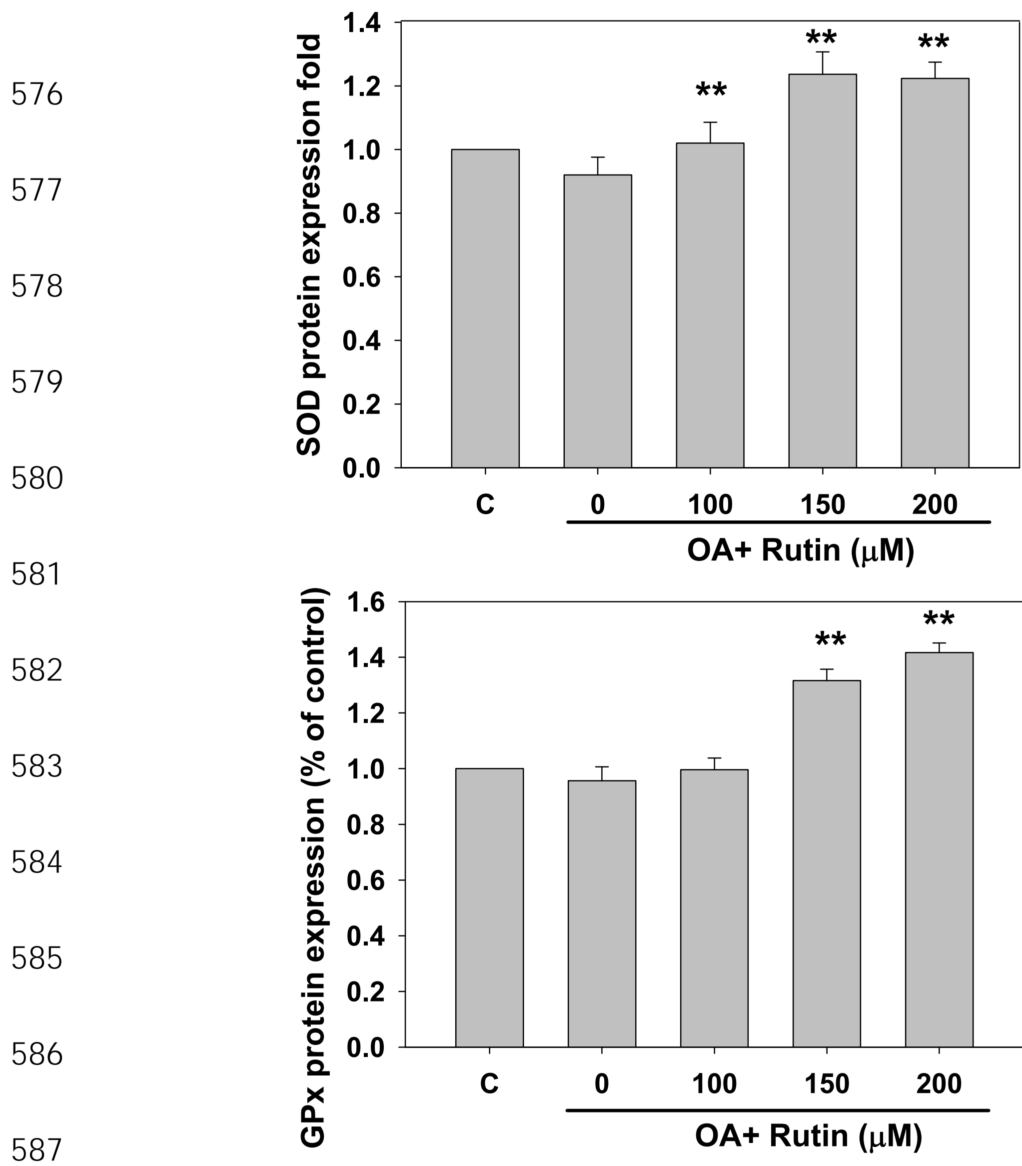

588 **Figure 5. Rutin increases the protein expression of anti-oxidant enzymes.** $HepG_2$
589 cells were exposed to the indicated concentrations of rutin in the presence of 600 μM
590 OA for 24 h. The catalase, GPx and SOD-1 protein expressiona were detected by
591 Western blot analysis. Data was representative of three independent experiments and
592 quantified by densitometric analysis. Expression levels were normalized to β-actin
593 protein level. The results from three repeated and separated experiments were similar
594 and expressed as mean ±SD. **, p<0.01.

595 **Figure 6.**

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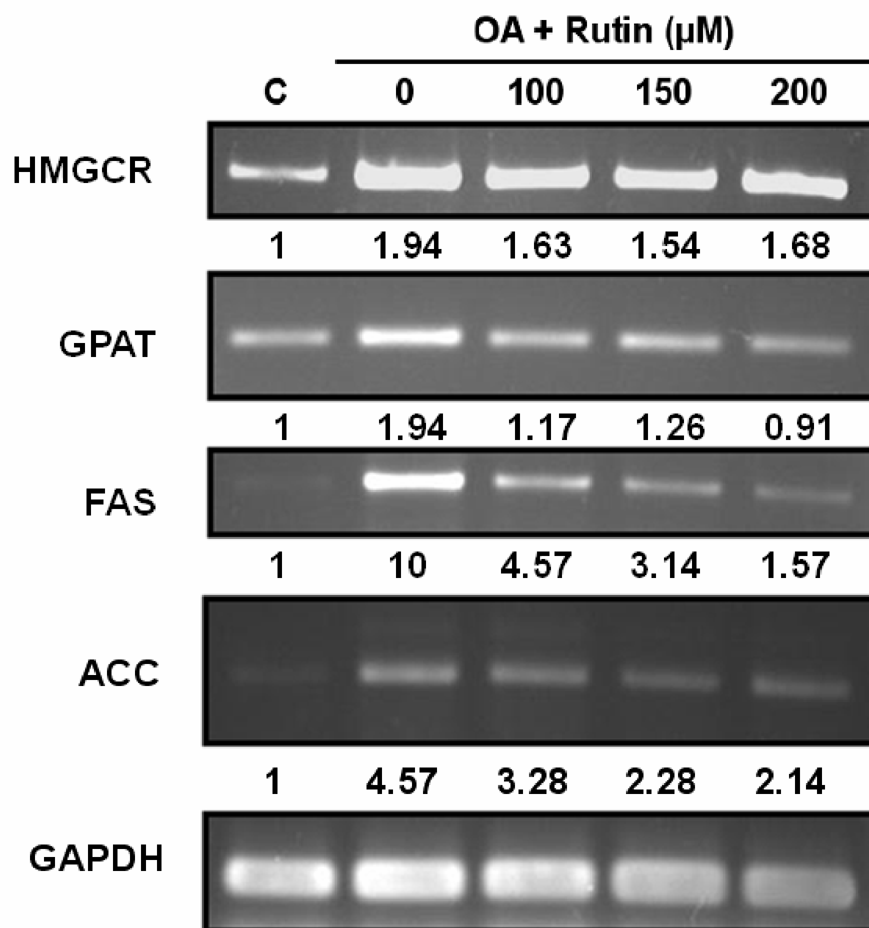

608 **Figure 6. Rutin decreases genes expression related to lipid metabolism in HepG2**
609 **cells.** The mRNAs of HMGCR, GPAT, FAS and ACC were extracted from cells
610 treated with the indicated concentrations of rutin in the presence of 600 μM OA for 24
611 h. The mRNA expression was analyzed by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

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