Molecular and cellular pharmacology

Carbenoxolone prevents the development of fatty liver in C57BL/6-Lep
\( \text{ob/ob} \) mice via the inhibition of sterol regulatory element binding protein-1c activity and apoptosis

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**A R T I C L E   I N F O**

Article history:
Received 7 February 2012
Received in revised form 11 June 2012
Accepted 13 June 2012
Available online 26 June 2012

Keywords:
Fatty liver
Sterol regulatory element binding protein-1c
Inflammation
Reactive oxygen species
Apoptosis

**A B S T R A C T**

Carbenoxolone is the 3-hemisuccinate of glycyrrhizinic acid, the active principal of licorice (Glycyrrhiza glabra). It was reported that carbenoxolone improved glucose tolerance with increased insulin sensitivity in mice with high fat diet-induced obesity. In the present study, we elucidated the protective effect of carbenoxolone in fatty liver animal models of C57BL/6-Lep\(^{\text{ob/ob}}\) mice through inhibition of hepatic lipogenesis and apoptosis. In addition, the potential mechanisms by which carbenoxolone could exert such protection were elucidated. Carbenoxolone was daily administered by gavage for 28 days in C57BL/6 and C57BL/6-Lep\(^{\text{ob/ob}}\) mice. Carbenoxolone prevented the plasma triglyceride and free fatty acid accumulation associated with the reduction of the expression of sterol regulatory element binding protein-1c, liver X receptor, fatty acid synthase and acetyl-CoA carboxylase in the livers of C57BL/6-Lep\(^{\text{ob/ob}}\) mice. Carbenoxolone also prevented hepatic injury through anti-apoptotic action in the livers of C57BL/6-Lep\(^{\text{ob/ob}}\) mice, accompanied by increased Bcl-2 expression and suppressed Bax and cytochrome c expression. As a mechanism, increased inflammatory cytokine expressions were inhibited by carbenoxolone in the fatty livers of C57BL/6-Lep\(^{\text{ob/ob}}\) mice. Furthermore, carbenoxolone inhibited free fatty acid (oleate/palmitate) induced reactive oxygen species formation and reversed free fatty acid induced mitochondrial membrane depolarization in HepG2 cells. Carbenoxolone prevents the development of fatty liver by inhibiting sterol regulatory element binding protein-1c expression and activity with an anti-apoptotic mechanism via the inhibition of inflammatory cytokine and reactive oxygen species formation in the livers of C57BL/6-Lep\(^{\text{ob/ob}}\) mice. It is suggested that carbenoxolone prevents the development and progression of fatty liver disease in patients with insulin resistance.

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1. Introduction

Evidence emerges that non-alcoholic fatty liver disease is associated with obesity and hepatic insulin resistance and progresses to non-alcoholic steatohepatitis (Angulo, 2002; Postic and Girard, 2008). The mechanism leading to non-alcoholic fatty liver disease remains unclear. At present time, two mechanisms have been proposed (Day, 2002). One is increased de novo lipogenesis and adipose lipolysis that lead to excess hepatic lipid accumulation (Postic and Girard, 2008). Sterol regulatory binding protein-1c is one of the major regulators of the expression of genes involved in hepatic triglyceride synthesis (Horton, 2002). Additionally, the activation of liver X receptor and specificity protein 1 increases sterol regulatory element binding protein-1c expression under insulin-stimulated conditions and leads to hepatic lipogenesis (Deng et al., 2007; Cagen et al., 2005). The other mechanism is oxidative stress-induced lipid peroxidation including hepatic injury and inflammation, provoking the progression toward non-alcoholic steatohepatitis (Day, 2002). The proinflammatory cytokine tumor necrosis factor-\( \alpha \) plays a central role in the etiology of non-alcoholic steatohepatitis by stimulating hepatic lipogenesis, and triggers hepatic mitochondrial dysfunction and oxidative stress that induce liver injury (Carter-Kent et al., 2008).

11\( \beta \)-hydroxysteroid dehydrogenases are microsomal enzymes belonging to the short-chain dehydrogenase/reductase family. 11\( \beta \)-hydroxysteroid dehydrogenase type 1 catalyzes the NADPH-dependent reduction of the 11-ketosteroids cortisone to cortisol. 11\( \beta \)-Hydroxysteroid dehydrogenase type 1 is highly expressed in liver, adipose tissue, skeletal muscle and macrophages. 11\( \beta \)-Hydroxysteroid dehydrogenase type 2 is a NAD\(^+\)-dependent dehydrogenase and catalyzes the oxidation of 11\( \beta \)-hydroxyglucocorticoids in kidney and colon. Recently, 11\( \beta \)-hydroxysteroid dehydrogenase...
2. Materials and methods

2.1. In vivo assay

2.1.1. Animals and drug administration

All animal experiments were carried out with 7–9-week-old male C57BL/6-Lep<sup>ob/ob</sup> mice (Korea Research Institute of Chemical Technology, Daejeon, Korea) according to the Guidelines for Animal Experimentation under admission of the Institutional Animal Care and Use committee (IACUC) of the Korea Research Institute of Chemical Technology. All animals were maintained in a room illuminated daily from 07:00 to 19:00 (12:12 h light/dark cycle), temperature (23 ± 1 °C), ventilation (10–12 times per hour), and humidity (55 ± 5%). Mice were caged individually and allowed free access to tap water and feed.

Carbenoxolone (10, 50 and 100 mg/kg body weight) was daily administrated by gavage at 17:00 to 18:00 for 28 days. Animals were weighed regularly to allow accurate dosing with drugs. The animals were sacrificed, and the liver was quickly removed and then frozen in liquid nitrogen. Food intake and body weight was measured weekly.

2.1.2. Histological analysis of liver

Liver tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline, and then transferred to 15% sucrose in phosphate-buffered saline. The tissues were frozen in an optical cutting temperature medium for frozen tissue specimens (Sakura Finetek Inc., Torrance, CA) by immersion in liquid nitrogen-equilibrated isopentane and then stored at −70 °C. Serial sections (4–6 μm thickness) were cut on a cryostat and stained with hematoxylin and eosin. Alternatively, intra-hepatic lipids were stained by the Oil red O method (Xu et al., 2009).

2.1.3. Plasma triglyceride and free fatty acid assay

For the triglyceride content assay and the free fatty acid concentration assay, plasma was separated by centrifu...
sulfate–polyacrylamide gel, and transferred to nitrocellulose membrane (Amerham Biosciences, Piscataway, NJ). Protein bands were visualized using chemiluminescence (Pierce, Rockford, IL). Polyclonal antibodies against Bax, cytochrome c, Actin and GAPDH, and monoclonal antibody against Bcl-2 were from the Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. In vitro assay

2.2.1. Cell culture

HepG2 (human hepatoma cell line) cells were cultured in Eagle’s minimum essential medium with 10% heat-inactivated fetal bovine serum and 1% antibiotics. Cells were grown to confluence at 37 °C in 5% CO₂.

2.2.2. Plasmids, transfection and reporter assays

Luciferase reporter constructs containing the wild-type sterol regulatory element binding protein-1c promoter (p sterol regulatory element binding protein (−1516/+40)-luciferase) was a kind gift from Dr. Kim (Seoul National University, Seoul, Korea). The synthetic specificity protein 1 promoter-reporter construct, pGL3–specificity protein 1, containing four specificity protein 1 binding sites (GC boxes), and the pCMV–specificity protein 1 expression plasmid were kindly given by Dr. Park (Keimyung University, Daegu, Korea).

For transfection of constructs, HepG2 cells were plated at a density of 4 × 10⁴ cells in a 100 mM culture dish and cultured for 24 h. The promoter-reporter constructs (36 µg/culture dish) were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were transfected for 5 h, washed to remove plasmid, and then maintained in a conditioned medium. HepG2 cells were plated at a density of 2 × 10⁴ per well in 96-well plate and cultured for 24 h. Cells were analyzed using the Steady-Glo luciferase assay system according to the manufacturer’s instructions (Promega, Madison, WI). Luciferase activity was detected using a SpectraMax M5e Microplate Reader (Molecular Devices, Menlo Park, CA).

2.2.3. Measurement of intracellular reactive oxygen species production and mitochondrial membrane potential

Cells were seeded with 5 × 10⁴ cells/well in 96-well tissue culture plates. The confluent cells were exposed to carbenoxolone for 24 h. Thereafter, 0.5 mM oleate:palmitate (2:1 ratio) was added for 24 h. Intracellular reactive oxygen species was measured by utilizing conversion of nonfluorescent 2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) into free dichlorofluorescin. Compound treated cells were incubated in the dark for 15 min in 50 mM phosphate buffer (pH 7.4) containing dichlorofluorescin diacetate. This agent is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the fluorescent polar derivative dichlorofluorescin and thereby trapped within the cells. The quantity of dichlorofluorescin fluorescence was measured at an emission wavelength of 545 nm and an excitation wavelength of 485 nm using a fluorescence plate reader (Bio-Tek Instruments, Inc. Winooski, VT). Results were expressed as percentage of control (nonstimulated cells) fluorescence intensity.

The mitochondrial membrane potential was measured by using the 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethyl-benzimidazolylcarbocyanine (JC-1; Molecular Probes, Eugene, OR). Cells were stained with 1 µg/ml of JC-1 for 30 min at 37 °C. JC-1 was excited at 488 nm and the monomer signal (green) was analyzed at 545 nm on a fluorescence plate reader (Bio-Tek Instruments, Inc. Winooski, VT). Simultaneously, the aggregate signal (red) was analyzed at 590 nm.

2.3. Drugs

Carbenoxolone was purchased from the Amifinecom Inc. (Petersburg, VA). Oleate, palmitate, Oil red O, NADPH and cortisone were from the Sigma–Aldrich (St. Louis, MO). Carbenoxolone was dissolved in dimethyl sulfoxide as a 20 mM stock solution and then diluted with the phosphate buffered saline.

2.4. Statistics

The results are expressed as means ± S.E.M. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test. *P < 0.05 was considered to be statistically significant.

3. Results

3.1. Carbenoxolone reduces lipid, triglyceride and free fatty acid accumulation in the livers of C57BL/6-Lepob/ob mice

Carbenoxolone reduced food intake and tended to decrease body weight. Liver weight of the C57BL/6-Lepob/ob mice treated with carbenoxolone was lower than that of vehicle mice (Table 1).

Histological examination showed that increasing amounts of large cytoplasmic lipid droplets were observed in the livers of C57BL/6-Lepob/ob mice compared with C57BL/6 mice. This change in cellular morphology was nearly completely prevented by 100 mg/kg carbenoxolone treatment (Fig. 1A). Moreover, triglyceride and free fatty acid concentration in the liver were significantly higher in C57BL/6-Lepob/ob mice than in C57BL/6 control mice. Administration of carbenoxolone (10, 50 and 100 mg/kg) dose-dependently decreased the concentrations of both triglyceride and free fatty acid (Fig. 1B and C), and also decreased 11β-hydroxysteroid dehydrogenase type 1 activity compared to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Metabolic Parameters of mice with or without carbenoxolone.</th>
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<tbody>
<tr>
<td></td>
<td>Body weight gain (g/28 days)</td>
</tr>
<tr>
<td>C57BL/6-control</td>
<td>3.82 ± 0.12</td>
</tr>
<tr>
<td>C57BL/6-carbenoxolone 100 mg/kg</td>
<td>3.97 ± 0.19</td>
</tr>
<tr>
<td>C57BL/6-Lepob/ob – vehicle</td>
<td>5.64 ± 0.33*</td>
</tr>
<tr>
<td>C57BL/6-Lepob/ob – carbenoxolone 10 mg/kg</td>
<td>5.50 ± 0.16</td>
</tr>
<tr>
<td>C57BL/6-Lepob/ob – carbenoxolone 50 mg/kg</td>
<td>5.49 ± 0.20</td>
</tr>
<tr>
<td>C57BL/6-Lepob/ob – carbenoxolone 100 mg/kg</td>
<td>5.20 ± 0.06</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n=4–9

* P < 0.001 vs. C57BL/6-control mice.

* P < 0.05 vs. C57BL/6-Lepob/ob-vehicle mice.
C57BL/6-Lep<sup>ob/ob</sup> vehicle mice (Fig. 1D). Thus, administration of carbenoxolone suppressed the fatty liver of C57BL/6-Lep<sup>ob/ob</sup> mice with suppression of triglyceride and free fatty acid concentration via inhibition of 11β-hydroxysteroid dehydrogenase type 1 activity.

### 3.2. Carbenoxolone reduces sterol regulatory element binding protein-1c expression and activity in the livers of C57BL/6-Lep<sup>ob/ob</sup> mice

To investigate whether sterol regulatory element binding protein-1c expression and activity are involved in the anti-adipogenic effects of carbenoxolone in the liver of C57BL/6-Lep<sup>ob/ob</sup> mice, we examined the mRNA sterol regulatory element binding protein-1c expression in liver and sterol regulatory element binding protein-1c activity in promoter-reporter construct transfected HepG2 cells.

Expression of sterol regulatory element binding protein-1c, a key adipogenic transcription factor, in the liver was significantly higher in C57BL/6-Lep<sup>ob/ob</sup> mice than in lean C57BL/6 mice. The administration of 100 mg/kg carbenoxolone significantly decreased sterol regulatory element binding protein-1c expression (P < 0.01) (Fig. 2A). The increased expression of other adipogenic transcription factors, liver X receptor α, fatty acid synthase and acetyl-CoA carboxylase 1, were also significantly reduced by the administration of carbenoxolone (Fig. 2B–D).

Furthermore, we studied the mechanism by which carbenoxolone inhibits sterol regulatory element binding protein-1c expression in the fatty liver. Transient transfection with a promoter-reporter construct in HepG2 cells showed that insulin and T0901317, a liver X receptor α agonist, significantly increased the sterol regulatory element binding protein-1c promoter activity, which was inhibited by 10 μM carbenoxolone (Fig. 3A).

A recent study showed that specificity protein 1 plays important role in insulin-stimulated sterol regulatory element binding protein-1c transcription (Cagén et al., 2005). Accordingly, we examined whether carbenoxolone inhibits insulin-stimulated specificity protein 1 transcriptional activity. Using a reporter construct composed of four synthetic specificity protein 1-response elements, we examined the effect of carbenoxolone on insulin-stimulated specificity protein 1 transactivating activity. Insulin (200 nM) induced a marked increase in specificity protein 1 promoter activity that was inhibited by carbenoxolone in a dose-dependent manner (Fig. 3B). Thus, carbenoxolone suppressed the fatty liver of C57BL/6-Lep<sup>ob/ob</sup> mice with suppression of sterol regulatory element binding protein-1c expression and activity via inhibition of specificity protein 1 transcriptional activity.

### 3.3. Carbenoxolone inhibits hepatic injury in C57BL/6-Lep<sup>ob/ob</sup> mice

The other mechanism leading to non-alcoholic fatty liver disease is oxidative stress-induced lipid peroxidation, including hepatic injury and inflammation, provoking the progression toward non-alcoholic steatohepatitis (Day, 2002). The effect of carbenoxolone on apoptosis was measured by using TUNEL analysis in the fatty livers of C57BL/6-Lep<sup>ob/ob</sup> mice. As shown in Fig. 4A, the number of TUNEL-positive cells significantly decreased in the livers of vehicle-treated C57BL/6-Lep<sup>ob/ob</sup> mice, which was markedly reduced by treatment with 100 mg/kg carbenoxolone. Carbenoxolone also elevated Bcl-2 expression, an anti-apoptotic protein, and suppressed Bax expression, pro-apoptotic protein in the fatty livers of C57BL/6-Lep<sup>ob/ob</sup> mice (Fig. 4B).

### 3.4. Carbenoxolone reduces inflammatory cytokine expression and reactive oxygen species formation in the livers of C57BL/6-Lep<sup>ob/ob</sup> mice

To identify the potential cellular mechanisms of the protective effect of carbenoxolone on hepatic injury of C57BL/6-Lep<sup>ob/ob</sup> mice, we examined the effect of carbenoxolone on the inflammatory cytokine expression by using real-time PCR in C57BL/6-Lep<sup>ob/ob</sup> mice. Inflammatory cytokine gene expression such as tumor necrosis factor-α and interleukin-6 significantly increased in the livers of vehicle-treated C57BL/6-Lep<sup>ob/ob</sup> mice, which was markedly reduced by the treatment with 50 and 100 mg/kg carbenoxolone (Fig. 5A and B).

To examine whether carbenoxolone was able to reduce oxidative stress in fatty liver, we measured reactive oxygen species concentrations in oleate/palmitate treated HepG2 cells, a validated in vitro model of steatosis recently reported by Gómez-Lechón et al. (2007). HepG2 cells loaded with oleate/palmitate (2:1 ratio) mimics benign chronic steatosis. 0.5 mM oleate/palmitate treated HepG2 cells reached similar levels of maximal intracellular lipid accumulation as found in human liver with steatosis. Therefore, we used 0.5 mM oleate/palmitate (2:1 ratio) in the present study (data not shown). Oleate/palmitate (0.5 mM) induced a marked increase in mRNA 11β-hydroxysteroid dehydrogenase type 1 expression (Fig. 6A). Furthermore, Oleate/palmitate induced increase of reactive oxygen species formation was significantly inhibited by carbenoxolone and the effect was dose-dependent (Fig. 6B). Carbenoxolone itself had no effect on reactive oxygen species production.

### 3.5. Carbenoxolone increase mitochondrial stability in HepG2 cells and C57BL/6-Lep<sup>ob/ob</sup> mice model

The results of Salvi et al. (2005) suggested that carbenoxolone failed the mitochondrial membrane potential and induced hydrogen peroxide generation, and thus could trigger the apoptotic pathway.

Thus, we examined on the effect of carbenoxolone on mitochondrial stability by measuring the mitochondrial membrane potential in HepG2 non-alcoholic fatty liver disease models and the cytosolic cytochrome c contents in the livers of C57BL/6-Lep<sup>ob/ob</sup> mice. Treatment with 0.5 mM oleate/palmitate for 24 h significantly decreased the mitochondrial membrane potential as indicated by the decrease of JC-1 aggregates and increase of JC-1 monomers (P < 0.001). After treatment with carbenoxolone for 24 h, the oleate/palmitate induced mitochondrial membrane depolarization was dose-dependently inhibited (P < 0.01, Fig. 7A).

The release of mitochondrial cytochrome c into cytosol is another indicator of perturbation of mitochondrial membrane stability. We further detected cytochrome c protein levels in cytosol using western blot analysis. Fatty liver of C57BL/6-Lep<sup>ob/ob</sup> mice increased the cytosolic cytochrome c level, which was inhibited by 100 mg/kg carbenoxolone (Fig. 7B). Thus, carbenoxolone prevented the development of fatty liver associated with anti-apoptotic mechanisms by inhibition of inflammatory cytokine expressions and reactive oxygen species formation and increased of mitochondrial stability.

### 4. Discussion

In the present study, we observed that carbenoxolone significantly inhibited the development of fatty liver in C57BL/6-Lep<sup>ob/ob</sup> mice through the inhibition of hepatic lipogenesis and apoptosis. The carbenoxolone-induced lipogenesis inhibitory effect was identified via the inhibition of sterol regulatory element binding
protein-1c expression and activity. Additionally, carbenoxolone inhibited the activity of specificity protein 1, which are a known mediators of insulin-dependent sterol regulatory element binding protein-1c expression (Cagen et al., 2005) and sterol regulatory element binding protein-1c related genes such as liver X receptor a, fatty acid synthase and acetyl-CoA carboxylase 1. In

Fig. 1. Inhibitory effect of carbenoxolone on lipid (A), triglyceride (B) and free fatty acid (C) accumulation via 11β-hydroxysteroid dehydrogenase type 1 inhibition (D) in the fatty livers of C57BL/6-Lepob/ob mice. Carbenoxolone (10, 50 and 100 mg/kg body weight) was administrated by gavage daily at 17:00 to 18:00 for 28 days in C57BL/6 and C57BL/6-Lepob/ob mice. Livers from each treatment group were fixed in formalin and subjected to hematoxylin and eosin (H&E) staining (b, upper panel) and oil-red O staining (b, lower panel). Lipid droplets in hepatocytes appear as darkly stained cytoplasm containing varying amounts of brownish red lipofuscin. The triglyceride and the free fatty acid content in plasma and the ex vivo 11β-hydroxysteroid dehydrogenase type 1 assay in liver tissues were carried out at 7 h after the last administration in vivo C57BL/6-Lepob/ob mice study. Results are expressed as means ± S.E.M. for n=4–9 mice per group. ###P < 0.001 vs. C57BL/6 vehicle group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. C57BL/6-Lepob/ob vehicle group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
conjunction with these, carbenoxolone inhibited oleate/palmitate-induced lipotoxicity via anti-apoptotic action and the reduction of reactive oxygen species formation.

The C57BL/6-Lep\textsuperscript{ob/ob} mice show severe insulin resistance with obesity due to a mutation in the leptin gene, and these mice have hepatic steatosis (Halaas et al., 1995). Inactivation of the sterol regulatory element binding protein-1 gene in the livers of C57BL/6-Lep\textsuperscript{ob/ob} mice was reported to show an approximately 50% reduction in hepatic triglycerides (Yahagi et al., 2002). Considering these reports, it is considered that sterol regulatory element binding protein-1 plays a significant role in the development of hepatic steatosis in this animal model of insulin resistance. Our present results showed that carbenoxolone reduces the histological fat deposition and triglyceride and free fatty acid

\begin{figure}[h]
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\caption{Inhibitory effects of carbenoxolone on sterol regulatory element binding protein-1c activity (A) and specificity protein 1 activity (B) in HepG2 cells. A. HepG2 cells were transfected with a p sterol regulatory element binding protein-1c (\textasciitilde{}1516\textasciitilde{}40)-luciferase construct vector or control empty vector and then stimulated with 200 nM insulin or 1 \mu M T0901317, a liver X receptor agonist, for 6 h, with or without 10 \mu M carbenoxolone pretreatment for 24 h. Results are expressed as means \pm S.E.M. of two experiments done with quadruplicate. ***P < 0.001 vs. control; ****P < 0.001 vs. 200 nM insulin or 1 \mu M T0901317 treated group. B. HepG2 cells were cotransfected with pCMV- specificity protein 1 (specificity protein 1) and the pG53- specificity protein 1 reporter (specificity protein 1) \times 4 luciferase) and then stimulated with 200 nM insulin for 6 h, with or without pretreatment with the indicated concentrations of carbenoxolone for 24 h. Results are expressed as means \pm S.E.M. of two experiments done with quadruplicate. ***P < 0.001 vs. control; **P < 0.01, ***P < 0.001 vs. 200 nM insulin treated group.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3.png}
\caption{Inhibitory effects of carbenoxolone on sterol regulatory element binding protein-1c luciferase activity (A) and specificity protein 1 luciferase activity (B) in HepG2 cells. A. HepG2 cells were transfected with a p sterol regulatory element binding protein-1c (\textasciitilde{}1516\textasciitilde{}40)-luciferase construct vector or control empty vector and then stimulated with 200 nM insulin or 1 \mu M T0901317, a liver X receptor agonist, for 6 h, with or without 10 \mu M carbenoxolone pretreatment for 24 h. Results are expressed as means \pm S.E.M. of two experiments done with quadruplicate. ***P < 0.001 vs. control; ****P < 0.001 vs. 200 nM insulin or 1 \mu M T0901317 treated group. B. HepG2 cells were cotransfected with pCMV- specificity protein 1 (specificity protein 1) and the pG53- specificity protein 1 reporter (specificity protein 1) \times 4 luciferase) and then stimulated with 200 nM insulin for 6 h, with or without pretreatment with the indicated concentrations of carbenoxolone for 24 h. Results are expressed as means \pm S.E.M. of two experiments done with quadruplicate. ***P < 0.001 vs. control; **P < 0.01, ***P < 0.001 vs. 200 nM insulin treated group.}
\end{figure}
concentrations in the livers of C57BL/6-Lepob/ob mice. In line with these, the results of Nuotio-Antar et al. (2007) showed that carbenoxolone inhibited atherosclerosis and hepatic steatosis with improvements in body composition and lipoprotein metabolism via 11β-hydroxysteroid dehydrogenase type 1 inhibition in obese and hyperlipidmic mice model. Under fasting conditions, activation of adenosine monophosphate–activated protein kinase was demonstrated to reduce lipogenesis in the liver by suppressing sterol regulatory element binding protein-1c activity (Park et al., 2008). Adenosine monophosphate–activated protein kinase activators, such as metformin and thiazolidinediones, have been shown to inhibit the expression of the sterol regulatory element binding protein-1c gene and to prevent the development of hepatic steatosis (Liu et al., 2010; Schroeder-Gloeckler et al., 2007). Sterol regulatory element binding protein-1c expression was also increased by the activation of liver X receptor α and specificity protein 1 under insulin-stimulated conditions and leads to hepatic lipogenesis (Deng et al., 2007; Cagen et al., 2005). Our results showed that while carbenoxolone did not inhibit the expression and phosphorylation of adenosine monophosphate–activated protein kinase (data not shown), carbenoxolone suppressed the sterol regulatory element binding protein-1c expression in fatty liver and the insulin-stimulated sterol regulatory element binding protein-1c activity in HepG2 cells. Carbenoxolone also inhibited sterol regulatory element binding protein-1c related genes such as liver X receptor α, a sterol regulatory element binding protein-1c upstream gene, and fatty acid synthase and acetyl-CoA carboxylase 1 and suppressed liver X receptor α agonist (T0901317)-stimulated sterol regulatory element binding protein-1c activation and insulin-stimulated specificity protein 1 activation. Considering these results with other reports, it is likely inferred that carbenoxolone inhibited hepatic lipogenesis via the inhibition of sterol regulatory element binding protein-1c expression and activity.

Steatotic livers are highly susceptible to oxidative stress. Oxidative stress induced lipid peroxidation, hepatic injury and inflammation, provoke the progression toward non-alcoholic steatohepatitis (Day, 2002). It is well established that the

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Fig. 4. Anti-apoptotic actions of carbenoxolone in the fatty liver of C57BL/6-Lepob/ob mice. (A) Mice were treated as described above. For the detection of cell death, in situ nick end labeling was performed using a TUNEL assay kit (QIA33; Oncogene, Boston, MA) according to product specifications and photographed (100 x ). (B) Mice were treated as described above and liver protein expression was determined by western blotting. Results are expressed as means ± S.E.M. for n = 4–9 mice per group. *P < 0.05, **P < 0.01 vs. C57BL/6 vehicle group; ***P < 0.001 vs. C57BL/6-Lepob/ob vehicle group.
oxidation of fatty acids is an important source of reactive oxygen species in fatty livers (Lieber, 2004; García-Ruiz et al., 1995; Hensley et al., 2000). The consequences of increased reactive oxygen species include the depletion of ATP and nicotinamide dinucleotide, DNA damage, the destruction of membranes via lipid peroxidation, and the release of proinflammatory cytokines (Brownning and Horton, 2004). The proinflammatory cytokine tumor necrosis factor-α plays a central role in the etiology of non-alcoholic steatohepatitis by stimulating hepatic lipogenesis and adipose lipolysis and triggers hepatic mitochondrial dysfunction and oxidative stress that induce liver injury and cell death (Carter-Kent et al., 2008; Browning and Horton, 2004). In line with these, inhibition of DNA fragmentation by carbenoxolone-suppression of inflammatory cytokines expression and reactive oxygen species production is a key mechanism of carbenoxolone-induced anti-apoptotic effects against hepatic injury in the C57BL/6-Lep⁻/⁻ mice.

Salvi et al. (2005) suggested that carbenoxolone failed the mitochondrial membrane potential and induced hydrogen peroxide generation when added at micromolar concentrations in liver mitochondria, and thus could trigger the apoptotic pathway. It was concluded that carbenoxolone enhance apoptosis by up-regulation of corticosterone availability via the inhibition of 11β-hydroxysteroid dehydrogenase and the induction of mitochondrial permeability transition indicating. The results of Pivato et al. (2006) also showed that carbenoxolone acts as an uncoupler of oxidative phosphorylation and, as an inhibitor of the ATP/ADP exchange system and the inhibitory action of carbenoxolone on mitochondrial energy metabolism could be contributing to induce the mitochondrial permeability transition, a key phenomenon in apoptosis. Furthermore, carbenoxolone induce liver damage during prolonged treatment (30 days) with daily doses of 150–300 mg (Laubenthal, 1972). However, treatment concentrations of carbenoxolone are likely to generate relatively high concentrations in the portal vein. Thus, we examined on the effect of carbenoxolone on mitochondrial stability by measuring the mitochondrial membrane potential in HepG2 non-alcoholic fatty liver disease models and the cytosolic cytochrome c contents in the livers of C57BL/6-Lep⁻/⁻ mice. However, carbenoxolone enhanced mitochondrial stability by measuring the membrane potential in HepG2 non-alcoholic fatty liver disease models and
inhibited cytosolic cytochrome c release in the fatty livers of C57BL/6-Lep\textsuperscript{ob/ob} mice at 100 mg/kg (in vivo) and 20 \mu M (in vitro).

Selective 11\beta-hydroxysteroid dehydrogenase type 1 inhibitors were reported to improve insulin sensitivity, glucose tolerance and lipid profiles (Morgan and Tomlinson, 2010). The suppression of 11\beta-hydroxysteroid dehydrogenase type 1 with RNA interference substantially inhibit 3T3-L1 adipogenesis (Liu et al., 2008b) and a novel selective 11\beta-hydroxysteroid dehydrogenase type 1 inhibitor prevents adipogenesis in human subcutaneous preadipocytes (Bujalska et al., 2008). Furthermore, induction of 11\beta-hydroxysteroid dehydrogenase type 1 activity and expression by inflammatory cytokines (tumor necrosis factor-\alpha, interleukin-6) may enhance orbital adipocyte differentiation (adipogenesis) in thyroid-associated ophthalmopathy (Tomlinson et al., 2010).

In our result, oleate/palmitate (0.5 mM) induced a marked increase in mRNA 11\beta-hydroxysteroid dehydrogenase type 1 expression and the 11\beta-hydroxysteroid dehydrogenase type 1 activity increased in the livers of C57BL/6-Lep\textsuperscript{ob/ob} mice compared with lean mice. However, the administration of carbenoxolone, a non-selective 11\beta-hydroxysteroid dehydrogenase inhibitor, significantly inhibited the 11\beta-hydroxysteroid dehydrogenase type 1 activity in the livers of C57BL/6-Lep\textsuperscript{ob/ob} mice.

5. Conclusion

Carbenoxolone, a non-selective 11\beta-hydroxysteroid dehydrogenase inhibitor, prevented the development of fatty liver associated with anti-apoptotic mechanisms by inhibition of inflammatory cytokine expressions and reactive oxygen species formation, and stimulated anti-lipogenesis action by inhibition of sterol regulatory element binding protein-1c expression and activity in the fatty livers of C57BL/6-Lep\textsuperscript{ob/ob} mice. This suggests that carbenoxolone can be a useful strategy to prevent the development and progression of non-alcoholic fatty liver disease in patients with insulin resistance.

Acknowledgements

This research was supported by the Center for Biological Modulators of the 21st Century Frontier R&D Program, the Ministry of Education, Science and Technology, and the Ministry of Knowledge Economy, Korea and the Korea Research Institute of Chemical Technology.

We greatly appreciate the gift of wild-type sterol regulatory element binding protein-1c promoter (p sterol regulatory element binding protein (-1516/+40)-luciferase) from Dr. Kim (Seoul National University, Seoul, Korea). The synthetic specificity protein 1 promoter-reporter construct, pG3-Sp1-function protein 1, containing four specificity protein 1 binding sites (GC boxes), and the pCMV-Sp1-function protein 1 expression plasmid were kindly given by Dr. Park (Keimyung University, Daegu, Korea).

References
