Carnosic acid attenuated acute ethanol-induced liver injury via a SIRT1/ p66Shc-mediated mitochondrial pathway

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Canadian Journal of Physiology and Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>cjpp-2015-0276.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>29-Aug-2015</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Tian, Xinyao; Central South University, Xiangya Hospital  
|                           | Hu, Yan; Second Affiliated Hospital of Dalian Medical University, Department of Pharmacy  
|                           | Li, Mingzhu; Dalian Medical University, Department of Pharmacology  
|                           | Xia, Kun; Central South University, Xiangya Hospital  
|                           | Yin, Jiye; Central South University, Clinical Pharmacology  
|                           | Chen, Juan; Central South University, Clinical Pharmacology  
|                           | Liu, Zhao-Qian; Institute of Clinical Pharmacology,  |
| Keyword:                  | ethanol, carnosic acid, SIRT1, p66shc, mitochondrial |

https://mc06.manuscriptcentral.com/cjpp-pubs
Title page

Carnosic acid attenuated acute ethanol-induced liver injury via a SIRT1/p66Shc-mediated mitochondrial pathway

Xinyao Tian¹, Yan Hu², Mingzhu Li³, Kun Xia¹, Jiye Yin⁴, Juan Chen⁴, Zhaoqian Liu⁴*

¹Xiangya Hospital, Central South University, Changsha 410008, P. R. China;
²Department of Pharmacy, Second Affiliated Hospital of Dalian Medical University, Dalian 116027, P. R. China;
³Department of Pharmacology, Dalian Medical University, Dalian 116044, P. R. China;
⁴Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008; P. R. China; Institute of Clinical Pharmacology, Central South University; Hunan Key Laboratory of Pharmacogenetics, Changsha 410078, P. R. China

*To whom correspondence should be addressed: Zhao-Qian Liu

Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008; Institute of Clinical Pharmacology, Central South University; Hunan Key Laboratory of Pharmacogenetics, Changsha 410078

Tel: +86 731 84805380

Fax: +86 731 82354476

E-mail: liuzhaoqian63@126.com (cj1028@csu.edu.cn)

https://mc06.manuscriptcentral.com/cjpp-pubs
Abstract

Ethanol-induced liver injury is associated with oxidative stress and hepatocyte apoptosis. We previously demonstrated that SIRT1/p66shc pathway activation attenuates hepatocyte apoptosis in liver ischemia/reperfusion. The current study aimed to investigate whether carnosic acid (CA), a natural antioxidant, can inhibit acute ethanol-induced apoptosis of hepatocytes and to determine the effect of SIRT1/p66Shc on this process. Our results showed that CA pretreatment significantly reduced ethanol-induced histologic damage, serum aminotransferase activity and oxidative stress in rats. Importantly, CA pretreatment increased SIRT1 expression following ethanol exposure. Furthermore, p66Shc expression was negatively correlated with SIRT1 expression. Consistent with the results demonstrating p66Shc inhibition, CA pretreatment inhibited the release of cytochrome C and apoptosis-inducing factor (AIF) from mitochondria. After exposing L02 cells to ethanol, the increased SIRT1 expression induced by CA was abrogated by pharmacologic SIRT1 inhibition or the use of siRNA against SIRT1. Additionally, SIRT1 inhibition significantly abrogated the suppression of p66Shc expression and mitochondrial translocation induced by CA. Accordingly, CA-induced decreases in the release of cytochrome C and AIF and in mitochondrial apoptosis were nearly abolished by SIRT1 knockdown. These data indicated that CA-activated SIRT1 is protective against ethanol treatment. In summary, CA attenuates acute ethanol-induced liver injury via a SIRT1/p66Shc-mediated mitochondrial pathway.

Keywords:
Introduction

Currently, alcohol abuse is a significant worldwide problem not only for individuals but also for society. The substantial morbidity and mortality associated with alcohol abuse has encouraged scientists to develop effective methods for postponing or preventing the pathogenesis that results from ethanol exposure (Holmes et al. 2014). Many studies indicate that ethanol metabolism can interrupt the mitochondrial redox balance that protects against reactive oxygen species (ROS). ROS have also been associated with inflammation and cell death in ethanol-induced liver injury. Therefore, designing a therapy to ameliorate ROS could be an important strategy for the treatment of ethanol-induced liver disease.

*Rosmarinus officinalis* L is an herb that is widely used as a folk medicine, in cosmetics and as a flavoring agent worldwide. A major compound of *R. officinalis* is carnosic acid (CA). CA has many pharmaceutical characteristics, such as antioxidative, anti-inflammatory and anti-adipogenic activities (Wang et al. 2012). Recently, there has been growing interest in its antioxidant mechanism. CA was shown to alleviate liver ischemia/reperfusion injury by suppressing ROS and apoptosis, while another study indicated that CA suppresses lipid peroxidation by inhibiting lipopolysaccharide-induced ROS (Xiang et al. 2013; Yan et al. 2014). However, studies that address the molecular mechanism of CA in acute ethanol-induced hepatic injury have not yet been conducted.
Alcohol abuse radically increases mitochondrial ROS production, overcoming the antioxidant defense system and leading to the pathogenesis of a number of human diseases. The impact of ROS and subsequent apoptosis can be inhibited, at least in part, by sirtuin 1 (SIRT1). SIRT1, a NAD-dependent class III histone deacetylase (HDAC), has been implicated in the regulation of genomic stability, cellular stress resistance, and senescence via the deacetylation of its target proteins (Vaziri et al. 2001). SIRT1 is the best-characterized target of ethanol in the liver, and it plays a significant role in alcoholic liver disease (Li et al. 2014; Yin et al. 2014).

The p66Shc protein belongs to a family of proteins that control cellular stress response and longevity, and it has been the topic of an emerging field of study: signal transmission by ROS. In response to pro-oxidant stimuli, p66Shc promotes the formation of ROS in mitochondria and stimulates apoptotic cell death. Among the potential mechanisms of alcohol-induced liver injury, p66Shc and ROS signaling appear to be challenging targets for specific modulation but useful for developing mitochondria-targeted therapeutics (Koch et al. 2008). Interestingly, we and others have recently reported that the inhibition of p66Shc expression by SIRT1 protects against hyperglycemia-induced endothelial damage and liver ischemia-reperfusion injury (Yan et al. 2014; Zhou et al. 2011). However, whether SIRT1-mediated p66Shc inhibition is implicated in acute alcoholic liver injury and by what exact mechanism this may occur are both currently unknown. According to the aims of this study, we considered the potential roles of cytochrome C and apoptosis-inducing factor (AIF) in these processes, as they are related to ethanol-induced mitochondrial dysfunction (X.
Zhang et al. 2010). This study aimed to evaluate the protective role of CA against ethanol-induced liver injury via a SIRT1/p66Shc-mediated mitochondrial pathway.

**Materials and methods**

**Reagents**

Carnosic acid (CA), purchased from Shanghai Winherb Medical Science Co., Ltd (Shanghai, China), was dissolved in olive oil for animal studies and in DMSO for cell culture experiments. Ethanol (purity >99%) was obtained from Sigma Co., Ltd (Sigma, USA).

**Animal model**

Male Wistar rats with weights ranging from 180 to 220 g were obtained from the Animal Center of Dalian Medical University (Dalian, China). The rats were housed under standard laboratory conditions for one week prior to experimentation. The experiments were performed in accordance with the institutional guidelines for the care and use of laboratory animals and were approved by the Committee on the Ethics of Animal Experiments of Dalian Medical University (Dalian, China).

The rats were pretreated with CA intragastrically (20 mg/kg, 40 mg/kg) once a day for three days. Ethanol, at a dose of 6 g/kg, was also administered intragastrically to the rats every 12 hours at 3 different time points to induce acute liver injury. The dosages of CA were determined in preliminary experiments. Rats in the control group were given an equal volume of olive oil as a vehicle control. Ten hours after the last alcohol
treatment, the rats were euthanized, and liver and blood samples were collected for further assays.

Biochemical assays

Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the manufacturer’s instructions, as were levels of glutathione (GSH), glutathione peroxidase (GSH-PX) and malondialdehyde (MDA) in the liver. All of the assay kits used were purchased from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China).

Liver histological examination

The middle lobe of the right liver of each rat was excised for histopathological examination. The liver samples were fixed in 4% neutral buffered formalin for 24 h, embedded in paraffin, sliced into 5-µm sections, stained with hematoxylin and eosin (H&E) and evaluated by examination with a light microscope.

Cell culture and cell viability assay

Human hepatic L02 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 mg/mL insulin. The cells were kept at 37°C in a humidified atmosphere of air and 5% CO₂. The protocol for each experiment is described in the corresponding figure legend. Cell viability and survival were determined using an MTT (Sigma) assay. The cells (1×10⁵) were plated
in 96-well microtiter plates and treated with different concentrations of CA or ethanol. After treatment, the cultures were incubated with an MTT solution (5 mg/ml) for 4 h at 37°C. The medium was then discarded, and formazan crystals were dissolved by the addition of 100 µl of dimethyl sulfoxide. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay microplate reader (Thermo, France). Cell viability was defined relative to an untreated control.

RNA Interference

The small interfering RNA (siRNA) sequences used to target SIRT1 are as follows: sense 5’-CCCUGUAAAGCUUUCAGAAdttdtf3’, antisense 5’-UUCUGAAAGCUUUCAGGdtdtdt-3’. Both specific and control siRNAs were obtained from GenePharma (Shanghai, China). After siRNA transfection for 48 hours, the cells were treated with 10 µM CA for an additional 6 hours. The cells were then collected for protein analysis.

Measurement of mitochondrial membrane integrity

Mitochondrial function was measured using a JC-1 membrane potential detection kit (Beyotime Institute of Biotechnology, Hangzhou, China). In healthy cells, a normal membrane potential enables the dye to enter and accumulate in the membrane, where it produces a red signal (excitation/emission 530 nm/590 nm). If membrane potential collapses (as in apoptotic cells), the dye remains in the cytosol, staining it green (excitation/emission 490 nm/530 nm). Thus, a decreased red/green fluorescence
intensity ratio reflects membrane depolarization during apoptosis. For JC-1 staining, the cells were washed once with PBS. JC-1 (4 µg/ml) was then immediately added to the plates, and the plates were incubated for 20 minutes at 37°C. The cells were then rinsed with PBS, and fresh PBS was added before fluorescence evaluation. Data analysis was performed using FlowJo software.

Western blot analysis

Nuclear, cytosolic, and mitochondrial protein fractions or total protein were prepared using a commercial protein isolation kit (KeyGEN Biotech, Nanjing, China). Equal amounts of protein from liver tissue and L02 cell homogenate were resolved by 10-15% SDS-PAGE (Bio-Rad, Hercules, USA) and subsequently transferred to a PVDF membrane (Millipore, Bedford, USA). For western blotting, primary antibodies against SIRT1, p66Shc, β-actin (Abcam Ltd., Cambridge, UK), voltage-dependent anion channels (VDAC, Proteintech Group, Wuhan, China), AIF and cytochrome C (Proteintech Group, Inc) were used. Appropriate secondary antibodies were used to detect the primary antibody/antigen complexes. The membranes were exposed to enhanced chemiluminescence-plus reagents (Beyotime Institute of Biotechnology, Jiangsu, China). Emitted light was detected with a multispectral imaging system (UVP, California, USA), and gels were analyzed with a Gel-Pro Analyzer, Version 4.0 (Media Cybernetics, Rockville, USA).

Real-time PCR analysis
Total RNA was extracted from the cultured cells or rat liver tissues using Trizol reagent (Life Technologies, Carlsbad, CA, USA). For real-time PCR analysis, cDNA was synthesized from the total RNA by reverse transcriptase (TAKARA, Dalian, China). The cDNA was subjected to real-time PCR analysis using SYBR® Premix Ex Taq (2×) (Tli RNaseH Plus), ROX plus* (TAKARA, Dalian, China). The specific gene expression primers for rats were 5’-CCCAGCTCCAGTCAGAACTAT-3’ (forward); 5’-TTGGCACCGATCCTCGAAC-3’ (reverse) for SIRT1; 5’-GACAGCACTGTGTTGGCATAG-3’ (forward); 5’-GGGACCTGACAGACTACCTC-3’ (reverse) for β-actin. The specific gene expression primers for cells were 5’-GCCTCACATGCAAGCTCTAGTGAC-3’ (forward); 5’-TTGGCAGGATCTGTGCCAATCATA-3’ (reverse) for SIRT1; 5’-CCAGCACAATGAAGATCAAG-3’ (forward); 5’-GTCGCCCTTATCCTTGAAGTAA-3’ (reverse) for β-actin. Relative mRNA abundance was obtained by normalizing to β-actin levels. Gene expression levels were calculated as $2^{-\Delta\Delta C_{\text{t}}}$ values. Each experiment was carried out three times.

Statistical analysis

All data were analyzed using SPSS 19.0 software. The results are expressed as the mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant. The data were analyzed with a two-tailed unpaired Student’s t-test or by one-way analysis of variance (ANOVA) to determine statistical significance between groups.
Results

Protective effect of CA against acute ethanol-induced liver injury

We first explored whether CA could protect rats from acute ethanol-induced hepatotoxicity. As shown in Fig. 1A and Fig. 1B, ethanol administration caused hepatotoxicity in rats, as indicated by increased serum ALT and AST levels. However, CA treatment significantly inhibited ethanol-induced ALT and AST activity in a dose-dependent manner, suggesting CA protects against ethanol-induced hepatotoxicity.

The protective effect of CA was further verified by histopathological analysis (Fig. 1C). The tissues of rats exposed to ethanol displayed increased cellular size, nuclear pleomorphism, cytoplasmic dissolution, apoptosis and accumulation of lipid droplets, which correlated with elevated serum levels of liver enzymes. In contrast, CA pretreatment dramatically reduced ethanol-induced liver damage. The cytoprotective effect of CA in vitro was then examined in an ethanol-treated L02 cell model. The exposure of L02 cells to ethanol resulted in a significant reduction of cell viability (Fig. 1D). Pretreatment of the cells with CA, however, attenuated the ethanol effect on cell viability in a dose-dependent manner. Thus, these results indicated that CA effectively protects against binge ethanol-induced liver injury.

Effect of CA on antioxidative activity during ethanol-induced hepatotoxicity

Ethanol metabolism generates ROS and causes lipid peroxidation, resulting in mitochondrial GSH depletion. These effects subsequently prime and sensitize
hepatocytes to injury, which may eventually lead to hepatocyte apoptosis (Farfan Labonne et al. 2009; Setshedhi et al. 2010). As shown in Fig. 2, binge ethanol-induced liver injury in rats caused a significant decrease in liver GSH and GSH-PX levels and a large increase in liver MDA levels compared with the control group. However, after pretreatment with CA, liver GSH and GSH-PX activity apparently increased, while liver MDA levels decreased.

CA-mediated protection against ethanol-induced hepatotoxicity associated with SIRT1 upregulation

SIRT1 activation has been shown to inhibit apoptosis and inflammation and to protect against oxidative stress, thereby improving acute and chronic liver injuries (Li et al. 2014; Yan et al. 2014; Yin et al. 2014). Hence, we investigated whether CA-mediated protection involves SIRT1 upregulation. As shown in Fig. 3A, after the administration of ethanol, hepatic SIRT1 protein levels were reduced in comparison to those of the control group. In contrast, CA pretreatment enhanced the expression of SIRT1 protein relative to the ethanol-only group in a dose-dependent manner. The level of SIRT1 mRNA was consistent with the level of SIRT1 protein expression (Fig. 3C). Thus, it appeared that SIRT1 activation was associated with CA-mediated protection against binge ethanol-induced hepatotoxicity.

Next, we used L02 cells to further confirm that CA treatment causes SIRT1 upregulation in vitro. In accordance with the in vivo results, SIRT1 protein levels in the ethanol group increased after a 10-µM CA pretreatment (Fig. 3B). However, the
activating effect of CA on SIRT1 was abolished when SIRT1 was inhibited by EX527, a specific SIRT1 inhibitor. We also examined SIRT1 mRNA expression in vitro. CA pretreatment increased SIRT1 mRNA expression in response to ethanol exposure (Fig. 3D). Whereas, the EX527 did not suppress SIRT1 mRNA expression, which is consistent with the results in HEK293 cells reported previously (Wu et al. 2012). Together, these results suggested that CA-mediated protection against ethanol-induced hepatotoxicity involves SIRT1 upregulation.

SIRT1 upregulation by CA inhibited p66Shc activation during binge ethanol-induced liver injury

SIRT1 and p66Shc have been implicated in ethanol-induced liver injury, having effects on lipid metabolism, ROS production, inflammation and apoptosis (Koch et al. 2008; Yin et al. 2014; Yin et al. 2012). Moreover, recent research has revealed that SIRT1-mediated inhibition of p66Shc expression protects against hyperglycemia-induced endothelial damage and liver ischemia-reperfusion injury (Yan et al. 2014; Zhou et al. 2011). Therefore, we aimed to determine whether SIRT1-mediated p66Shc inhibition was involved in binge alcohol-induced liver injury. As expected, p66Shc protein levels in the liver were significantly increased in the ethanol group compared to the control group. However, CA pretreatment effectively repressed p66Shc protein expression in a dose-dependent manner (Fig. 4A). To assess whether ethanol increases mitochondrial translocation of p66Shc, the presence of p66Shc in mitochondrial lysates was assessed by western blotting. As shown in Fig.
4C, after ethanol treatment, mitochondrial p66Shc expression significantly increased, but CA pretreatment inhibited p66Shc mitochondrial translocation in a dose-dependent manner.

*In vitro* studies were used to further explore the role of SIRT1 in regulating p66Shc during ethanol-induced hepatotoxicity. The results showed that the inhibition of SIRT1 by EX527 resulted in increased p66Shc expression and mitochondrial translocation in L02 cells after ethanol exposure (Fig. 4B). Moreover, CA-induced downregulation of p66Shc expression and mitochondrial translocation was attenuated by SIRT1 knockdown. Together, these results indicated that SIRT1-mediated p66Shc inhibition plays a role in binge alcohol-induced liver injury and that the protective effects of CA against alcohol-induced liver injury may be related to this mechanism.

CA alleviated binge ethanol-induced mitochondrial injury

In mitochondria, p66Shc oxidizes cytochrome C to generate ROS, which induce the opening of the mitochondrial permeability transition pore, thereby facilitating the release of cytochrome C and H$_2$O$_2$ into the cytosol (Giorgio et al. 2005). Thus, western blot analysis was used to assess isolated mitochondrial and cytosolic fractions to identify the subcellular localizations of proteins involved in the mitochondria-mediated apoptotic signaling pathway. In the liver, ethanol exposure caused a significant release of cytochrome C from mitochondria into the cytosol. In contrast, accompanying SIRT1-mediated p66Shc inhibition, CA pretreatment substantially restored the mitochondrial localization of cytochrome C (Fig. 5A).
further verify this observation, we examined cytochrome C release *in vitro* (Fig. 5B). After ethanol treatment, CA-treated cells showed a decrease in the release of cytochrome C; however, cells treated with EX527 showed a clear increase in the release of cytochrome C from mitochondria. Furthermore, EX527 significantly attenuated CA-mediated inhibition of cytochrome C release. These results indicated that the protective effects of CA against ethanol-induced liver injury may be related to decreased release of cytochrome C. Mitochondrial damage can also release AIF, another important mitochondrial protein. AIF released from mitochondria is subsequently translocated to the nucleus; this pathway is a typical feature of apoptotic death (Cherian et al. 2008). As with cytochrome C, AIF was released from mitochondria into the nucleus after ethanol treatment (Fig. 5C). Moreover, AIF release could be reduced by CA or aggravated by EX527 (Fig. 5D).

Ethanol not only triggers mitochondrial superoxide production but also disrupts the mitochondrial membrane (Xu et al. 2015). Therefore, JC-1 staining, a method of assessing mitochondrial damage, was used *in vitro* (Fig. 5E). As expected, ethanol caused a significant loss of JC-1 red fluorescence and led to increased green fluorescence relative to the control, indicating apoptosis in the mitochondria; however, CA treatment nearly completely reversed these changes. In contrast, EX527 markedly attenuated CA-mediated inhibition of these mitochondrial membrane changes. Collectively, these findings suggest that CA alleviates binge ethanol-induced injury, which may partially depend on a SIRT1/p66Shc-mediated mitochondrial pathway.

To further investigate the effects of SIRT1/p66Shc on mitochondria during ethanol
exposure, SIRT1 was silenced using siRNA (si-SIRT1). si-SIRT1 increased both p66Shc expression and the release of cytochrome-c and AIF from mitochondria (Fig. 6). In addition, si-SIRT1 abolished the inhibition of p66Shc expression and the release of cytochrome-c and AIF caused by treatment with CA. Taken together, these results demonstrate that CA protects against ethanol-induced mitochondrial injury in a process that involves SIRT1-mediated p66Shc inhibition.

**Discussion**

Alcohol abuse is a worldwide public health issue. The molecular mechanisms underlying acute ethanol hepatotoxicity are still debated. Therefore, further information is urgently needed to identify novel preventive and therapeutic opportunities. The present work showed that SIRT1/p66Shc is a key therapeutic target for preventing binge ethanol-induced liver injury and that CA effectively protects against this injury type, which may be associated with a SIRT1/p66Shc-mediated mitochondrial pathway.

Rosemary is a plant that is widely utilized in the food industry for its functional properties and beneficial health properties. CA exhibits a range of pharmaceutical properties, including antioxidant, anti-inflammatory and anti-apoptotic effects (Wang et al. 2011; Wang et al. 2012). In the present study, we explored the protective role of CA against binge ethanol-induced liver injury. We found that serum transaminase activity markedly increased following acute alcohol intoxication. In parallel with this effect, enhanced lipid peroxidation and decreased GSH and GSH-PX levels were
observed in the liver. However, CA pretreatment effectively restored antioxidant enzyme activity and protected the liver from alcohol-induced injury.

Among the seven sirtuin genes found in mammals, SIRT1, a homolog of SIR2 in the yeast *Saccharomyces cerevisiae*, has been the most extensively studied. SIRT1 is highly expressed in the liver, where it appears to be involved in maintaining normal liver function. Reduced expression of hepatic SIRT1 predisposes mice to high-fat diet-induced hepatic steatosis (Purushotham et al. 2009). Additionally, SIRT1 plays a central role in the pathogenesis of alcoholic liver disease. Recent reports have shown that alcohol consumption decreases SIRT1 expression, and hepatocyte-specific deletion of SIRT1 causes hepatic steatosis and inflammation (Li et al. 2014; Yin et al. 2014; Yin et al. 2012). In support of these observations, we found that SIRT1 expression in the liver significantly decreased in rats acutely exposed to alcohol. However, CA enhanced SIRT1 expression, indicating that CA might protect rats from acute alcohol-induced liver injury via SIRT1 upregulation. This idea was further confirmed by SIRT1 inhibition in L02 cells. In ethanol-exposed cells, CA pretreatment increased SIRT1 expression; however, specific inhibition of SIRT1 by EX527 or si-SIRT1 attenuated this upregulation, indicating that CA’s effects on SIRT1 provide protection against acute ethanol-induced liver injury.

A previous study indicated that p66Shc is a potential molecular mediator of the deleterious effects of alcohol on cell metabolism and viability and that it plays a vital role in mitochondrial damage in alcohol-dependent pathologies. In our study, p66Shc expression significantly increased following ethanol exposure, while CA pretreatment
caused it to dose-dependently decrease. Furthermore, p66Shc underwent mitochondrial translocation following ethanol exposure, which was restrained by CA. This result indicated that the protection against ethanol-induced liver injury afforded by CA treatment involved its inhibitory effect on p66Shc. A growing body of evidence has implied that SIRT1 is crucial for repressing p66Shc transcription (Yan et al. 2014; Zhou et al. 2011). Zhou et al. demonstrated that SIRT1 binds to the p66Shc promoter (508 bp to 250 bp), resulting in decreased acetylation of p66Shc promoter-bound histone H3. As a result, SIRT1 overexpression was found to decrease high glucose-induced p66Shc expression in human umbilical vein endothelial cells, an effect that was accompanied by reduced oxidative stress and improved endothelial function (Zhou et al. 2011). Consistent with this observation, our study demonstrated that variations in p66Shc expression were opposite to those in SIRT1 expression, indicating that SIRT1 may inhibit p66Shc expression upon ethanol exposure. In vitro experiments with EX527 and si-SIRT1 provided further evidence to support this argument. In cells, the inhibition of SIRT1 by EX527 or si-SIRT1 induced p66Shc expression and attenuated the inhibition of p66Shc expression caused by CA (Fig. 4). Together, these observations demonstrated that CA-mediated inhibition of p66Shc occurs at least partially through the upregulation of SIRT1 expression during acute ethanol-induced liver injury.

Cytochrome C and AIF are pro-apoptotic proteins that are normally restricted to the mitochondria, and their release during the apoptotic cascade has been demonstrated (Gustafsson and Gottlieb 2008; Phaneuf and Leeuwenburgh 2002; Susin et al. 1999).
Cytochrome C is released into the cytosol, where it triggers caspase-3 activation during oxidization, resulting in apoptosis (Giorgio et al. 2005; Kluck et al. 1997; Seo et al. 2013). Similarly, the translocation of AIF, a 67-kD flavoprotein, from the mitochondria to the nucleus results in a “caspase-independent” pathway for programmed cell death (Cherian et al. 2008; Yu et al. 2006). Importantly, mutations in p66Shc impair its ability to mediate apoptosis by suppressing its interaction with mitochondrial cytochrome C (Giorgio et al. 2005). Conversely, an absence of SIRT1 leads to AIF-mediated cell death (Kolthur-Seetharam et al. 2006). In the present study, we estimated the release and accumulation of the mitochondrial proteins cytochrome C and AIF into the cytosol. Our study revealed that CA pretreatment significantly inhibited the release of cytochrome C and AIF from liver mitochondria after ethanol exposure. In contrast, CA-induced inhibition of cytochrome C and AIF release from mitochondria was blocked when SIRT1 was inhibited by EX527 or si-SIRT1. Meanwhile, JC-1 analysis of mitochondrial membrane integrity showed that mitochondrial injury in L02 cells was attenuated by CA but aggravated by EX527. Together with data suggesting that disturbances in mitochondrial membrane integrity are essential for ethanol-induced cell death, the present data suggest that a CA-responsive SIRT1/p66Shc pathway has the potential to ameliorate ethanol-induced mitochondrial oxidative stress, leading to a subsequent decrease in pathological damage (Adachi and Ishii 2002; Koch et al. 2004; P. Zhang et al. 2015). In summary, we demonstrated for the first time that CA protects the liver from binge ethanol-induced liver injury through a SIRT1/p66Shc-mediated mitochondrial...
pathway. These findings provide rationale for the potential clinical application of CA for the prevention or treatment of liver intoxication.

Acknowledgements

We appreciate the support provided by the National High-tech R&D Program of China (863 Program) (2012AA02A517), the National Natural Science Foundation of China (81173129, 81202595, 81373490), and the Fundamental Research Funds for the Central Universities of Central South University.

References


Gustafsson, A. B., and Gottlieb, R. A. 2008. Heart mitochondria: gates of life and


Nature, 397(6718): 441-446.


Figure legends

Figure 1  CA diminishes binge ethanol-induced liver injury. Rats were treated with CA before ethanol was administered. (A) Serum ALT level. (B) Serum AST level. (C) Liver histopathology by H&E staining and histologic injury scores in groups. a, Control; b, Control + CA (40 mg/kg); c, Ethanol (6 g/kg); d, Ethanol + CA (20 mg/kg); and e, Ethanol + CA (40 mg/kg). H&E-stained sections were photographed at 400 × magnification. (D). Cell viability. L02 cells were pretreated with CA (2.5, 5 and 10 μM) for 6 h and were then exposed to 100 mM ethanol for 24 h; cell viability was determined by MTT assay. Results are expressed as the mean ± SD (n = 6). **p < 0.01 vs. control group, #p < 0.01 vs. ethanol group.

Figure 2  CA improves hepatic antioxidative activity. (A) The hepatic GSH level in rats. (B) The hepatic GSH-PX level in rats. (C) The hepatic MDA level in rats. Results are expressed as the mean ± SD (n = 6). **p < 0.01 vs. control group, #p < 0.05 vs. ethanol group, #p < 0.01 vs. ethanol group.

Figure 3  Effects of CA on SIRT1 expression in vivo and in vitro. (A) Protein expression of SIRT1 in rats. (B) Protein expression of SIRT1 in L02 cells. L02 cells were treated with or without 10 μM EX527 for 6 h, were incubated with 10 μM CA for a further 6 h, and were then exposed to 100 mM ethanol for 24 h. (C) Messenger RNA (mRNA) expression of SIRT1 in rats. (D) Messenger RNA (mRNA) expression of SIRT1 in cells. β-actin was used to normalize protein levels. **p < 0.01 vs. control.
group, $p < 0.05$ vs. ethanol group, $p < 0.01$ vs. ethanol group, $p < 0.01$ vs. ethanol + CA group ($n = 3$).

**Figure 4** Activation of SIRT1 by CA inhibits binge ethanol-induced upregulation of p66Shc in vivo and in vitro. (A) Protein expression of p66Shc in rats. (B) Protein expression of p66Shc in L02 cells. L02 cells were treated with or without 10 µM EX527 for 6 h, were incubated with 10 µM CA for 6 h, and were then exposed to 100 mM ethanol for 24 h. (C) Mitochondrial expression of p66shc in rats. (D) Mitochondrial expression of p66shc in L02 cells. β-actin was used to normalize the total of p66Shc protein levels. VDAC was used to normalize mitochondrial p66Shc protein levels. *$p < 0.05$ vs. control group, **$p < 0.01$ vs. control group, $p < 0.05$ vs. ethanol group, $p < 0.01$ vs. ethanol group, $p < 0.01$ vs. ethanol + CA group ($n = 3$).

**Figure 5** CA alleviates binge ethanol-induced mitochondrial injury. (A-B) Protein expression of cytoplasmic and mitochondrial cytochrome C in rats and in L02 cells. (C-D) Protein expression of nuclear and mitochondrial AIF in rats and in L02 cells. L02 cells were treated with or without 10 µM EX527 for 6 h, were incubated with 10 µM CA for 6 h, and were then exposed to 100 mM ethanol for 24 h. *$p < 0.05$ vs. control group, **$p < 0.01$ vs. control group, $p < 0.05$ vs. ethanol group, $p < 0.01$ vs. ethanol group, $p < 0.01$ vs. ethanol + CA group ($n = 3$). (E) Mitochondrial membrane integrity assessment in L02 cells using JC-1 analysis. a, Control; b,
Control + CA (10 µM); c, Ethanol (100 mM); d, Ethanol + CA (10 µM); e, Ethanol + EX527 (10 µM); and f, Ethanol + EX527 (10 µM) + CA (10 µM).

**Figure 6** Si-SIRT1 attenuated the protective effect of CA on mitochondrial injury induced by ethanol. (A) Protein expression of SIRT1 in L02 cells. (B) Protein expression of p66Shc in L02 cells. (C) Protein expression of cytoplasmic and mitochondrial cytochrome C in L02 cells. (D) Protein expression of nuclear and mitochondrial AIF in L02 cells. L02 cells were cultured, transfected with control or SIRT1 siRNA, and exposed to 10 µM CA for 6 h. The cells were then exposed to 100 mM ethanol for 24 h. β-actin was used to normalize protein levels. Values are means ± SD, n = 3. *p < 0.05 vs. ethanol group, **p < 0.01 vs. ethanol group (n = 3).
Figure 1  CA diminishes binge ethanol-induced liver injury. Rats were treated with CA before ethanol was administered. (A) Serum ALT level. (B) Serum AST level. (C) Liver histopathology by H&E staining and histologic injury scores in groups. a, Control; b, Control + CA (40 mg/kg); c, Ethanol (6 g/kg); d, Ethanol + CA (20 mg/kg); and e, Ethanol + CA (40 mg/kg). H&E-stained sections were photographed at 400 × magnification. (D). Cell viability. L02 cells were pretreated with CA (2.5, 5 and 10 µM) for 6 h and were then exposed to 100 mM ethanol for 24 h; cell viability was determined by MTT assay. Results are expressed as the mean ± SD (n = 6). **p < 0.01 vs. control group, ##p < 0.01 vs. ethanol group.
Figure 2  CA improves hepatic antioxidative activity. (A) The hepatic GSH level in rats. (B) The hepatic GSH-PX level in rats. (C) The hepatic MDA level in rats. Results are expressed as the mean ± SD (n = 6). **p < 0.01 vs. control group, #p < 0.05 vs. ethanol group, ##p < 0.01 vs. ethanol group.