Salvianolic acid B protects against acetaminophen hepatotoxicity by inducing Nrf2 and phase II detoxification gene expression via activation of the PI3K and PKC signaling pathways

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ABSTRACT

Acetaminophen (APAP) is used drugs worldwide for treating pain and fever. However, APAP overdose is the principal cause of acute liver failure in Western countries. Salvianolic acid B (SalB), a major water-soluble compound extracted from Radix Salvia miltiorrhiza, has well-known antioxidant and anti-inflammatory actions. We aimed to evaluate the ability of SalB to protect against APAP-induced acute hepatotoxicity by inducing nuclear factor-erythroid-2-related factor 2 (Nrf2) expression. SalB pretreatment ameliorated acute liver injury caused by APAP, as indicated by blood aspartate transaminase levels and histological findings. Moreover, SalB pretreatment increased the expression of Nrf2, Heme oxygenase-1 (HO-1) and glutamate-cysteine ligase catalytic subunit (GCLC). Furthermore, the HO-1 inhibitor zinc protoporphyrin and the GCLC inhibitor buthionine sulfoximine reversed the protective effect of SalB. Additionally, siRNA-mediated depletion of Nrf2 reduced the induction of HO-1 and GCLC by SalB, and SalB pretreatment activated the phosphatidylinositol-3-kinase (PI3K) and protein kinase C (PKC) signaling pathways. Both inhibitors (PI3K and PKC) blocked the protective effect of SalB against APAP-induced cell death, abolishing the SalB-induced Nrf2 activation and decreasing HO-1 and GCLC expression. These results indicated that SalB induces Nrf2, HO-1 and GCLC expression via activation of the PI3K and PKC pathways, thereby protecting against APAP-induced liver injury.

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1. Introduction

Acute liver failure is a global disease that can be attributed to various liver lesions. The major causes of acute liver failure are viral infection, drug exposure, accidental food poisoning and radiation damage (1,2). Acetaminophen (APAP) is widely used as an analgesic and antipyretic agent; it is also known to induce liver injury, accounting for the most common form of acute liver failure (3). Toxicity is initiated by cytochrome P450-mediated metabolism of APAP to the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI). The binding of NAPQI to cellular macromolecules induces cellular oxidant stress and DNA damage, leading to severe centrilobular hepatotoxicity and acute liver failure (4,5). Therefore, designing therapies that inhibit oxidative stress and scavenge electrophiles is expected to be one of the most important strategies for treating APAP-induced liver injury.
Nuclear factor-erythroid-2-related factor 2 (Nrf2) plays a central role in the induction of antioxidant enzymes via binding to antioxidant response elements (AREs). This regulation can be grouped into several categories according to the induction of phase II detoxifying enzymes, antioxidant genes, scavenger receptor and transporters (6). Furthermore, among the antioxidant and detoxification enzymes, Heme oxygenase-1 (HO-1) and glutamate-γ-cysteine ligase catalytic subunit (GCLC) are the most important. Recent studies have shown that activation of Nrf2 plays an important role in acute liver injury both in vitro and in vivo (7,8). To date, multiple signaling kinases have been reported to regulate Nrf2, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) (9,10).

Salvianolic acid B (SalB) is the most abundant and bioactive component of salvianolic acid extracted from Radix Salvia miltiorrhiza, which is officially listed in the Chinese Pharmacopoeia (11). Previous research has demonstrated that SalB inhibits lipid per-oxidation and scavenges superoxide anions and hydroxyl radicals both in vitro and in vivo (12,13). There is evidence that treatment with SalB reduces brain damage and improves motor function after cerebral ischemia in rats (14,15). In addition, SalB has been reported with SalB reduces brain damage and improves motor function after in vitro.

Previous research has demonstrated that SalB inhibits lipid per-oxidation and scavenges superoxide anions and hydroxyl radicals both in vitro and in vivo (12,13). There is evidence that treatment with SalB reduces brain damage and improves motor function after cerebral ischemia in rats (14,15). In addition, SalB has been reported to protect against liver fibrosis in patients and animals, as well as inhibit CCl4-induced hepatic fibrosis; these beneficial effects are associated with the regulation of NF-κB(κB/κα) signaling (16,17).

The above observations regarding the biological activities of SalB compelled us to investigate whether SalB can attenuate APAP-induced liver injury. The goals of this study were to investigate the expression of Nrf2 in vivo. To date, multiple signaling kinases have been reported to regulate Nrf2, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K) (9,10).

2. Materials and methods

2.1. Chemicals

SalB with a purity >98% was obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetaminophen and dimethyl sulfoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Eagle’s minimum essential medium (MEM) and trypsin were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). RNase inhibitor and fetal bovine serum at 37 °C was purchased from Alfa Aesar (Ward Hill, MA, USA). PD98059, SB203580, SP600125 and GF109203X, as well as buthionine sulfoximine (BSO), were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Zinc protoporphyrin (ZnP) was purchased from Alfa Aesar (Ward Hill, MA, USA).

2.2. Animals

Male Kunming mice, weighing 18–22 g, were provided by the Animal Center of Dalian Medical University (Dalian, China). The animals were fed standard chow and water, housed in plastic cages and maintained at 22 ± 2 °C with 50–60% relative humidity and a 12-h light–dark cycle throughout the experiment. All procedures were conducted according to the institutional animal care guidelines and approved by the Institutional Ethics Committee.

Animals were divided into five groups of ten animals per group. Group 1 received vehicle (double-distilled water) only and served as the normal control. Group 2 only received 50 mg/kg SalB (dissolved in double-distilled water) once a day for three days. Group 3 animals were intragastrically administered a single dose of APAP at 300 mg/kg (dissolved in double-distilled water at 37 °C) and then kept as the experimental model. Groups 4 and 5 received SalB at 25 and 50 mg/kg, respectively, once a day for three consecutive days. Two hours after the last SalB pretreatment, the animals received an intragastric dose of APAP. The experimental animals were sacrificed, and blood samples were collected from the abdominal aorta 24 h after APAP administration. The serum was separated by centrifugation at 3000 rpm/min for 30 min at 4 °C. The liver was collected from each mouse for biochemical, Western blot, and histopathological analysis.

2.3. Cell culture

Well-differentiated human hepatoma cell line HepG2 has been used to identify factors which regulate hepatic gene expression during the host response to tissue injury (18). And in Guerrero JA et.al study they found that using anoikis-hepatocytes from primary culture and H2O2-treated HepG2 cells confirmed the presence of a similar complex (19). So in this study, we used HepG2 cells as our model cells to explore the mechanism of SalB in APAP-induced injury.

HepG2 cells were cultured in MEM supplemented with 10% (v/v) fetal bovine serum at 37 °C in a 5% CO2 humidified incubator. Cells seeded at a density of 1 × 10⁵ cells per well were grown for 24 h and then treated with SalB at various concentrations (0.5, 2, 8 μmol/L). Each experimental protocol is described in the corresponding figure legends.

2.4. Biochemical index assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) represent the liver function along with disease and are the most commonly used biochemical markers. Mouse serum ALT and AST levels in liver tissues were measured using an assay kit (Nanjing Jiancheng Corp., Nanjing, China) according to the manufacturer’s recommendations.

2.5. Histopathological evaluation

Fresh hepatic samples were fixed in 10% neutral formalin for 24–48 h before being embedded in paraffin. After embedding, the samples were cut into 5-μm sections and mounted onto slides. Duplicate sections were stained with hematoxylin and eosin (H&E) for pathological evaluation.

2.6. Western blot analysis

Cytoplasmic and nuclear proteins were extracted from HepG2 cells or liver tissue using a protein extraction kit (KeyGen Biotech, Nanjing, China). Extracted proteins were separated using SDS-PAGE and immunoblotted with the indicated primary antibodies. The blots were then incubated with secondary antibodies. The membranes were exposed to enhanced chemiluminescence-plus reagents (Beyotime Institute of Biotechnology, Hangzhou, China), documented using a BioSpectrum-410 multispectral imaging system with a Chemi HR camera 410 and analyzed using the Gel-Pro Analyzer, version 4.0 (Media Cybernetics, Rockville, MD, USA).

2.7. RNA isolation and RT-PCR

Total RNA was extracted from HepG2 cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the TaKaRa RNA PCR Kit (AMV) Version 3.0 (TaKaRa, Shiga, Japan). For PCR analysis, the primers and their sequences were reported in our previous study (20). The co-amplified PCR products were separated on a 1.5% agarose gel using
electrophoresis. A BioSpectrum-410 multispectral imaging system with a Chemi HR camera 410 (UVP Inc., Upland, CA, USA) was used to analyze the intensity of the DNA bands.

2.8. Transient transfection and RNA interference

HepG2 cells were transfected with specific siRNA at a concentration of 100 nM using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Human Nrf2 siRNA was obtained from GenePharma Technology (Shanghai, China). After siRNA transfection, cells were incubated with fresh culture medium containing 8 μM SalB. The protein extracts made from the siRNA transfection experiments were analyzed to determine the expression of HO-1, GCLC, and Nrf2 via Western blotting.

2.9. Statistical analysis

Data are expressed as the mean ± SD. The significance of the differences among the mean values was evaluated using one-way analysis of variance. The Student-Newman-Keuls (SNK)/least significant difference (LSD) test was applied to make pairwise comparisons among the means. Differences were considered statistically significant when the p value was less than 0.05. All statistical analyses were performed using the SPSS16 statistical software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. SalB protects against APAP-induced acute liver injury

We first determined the protective effect of SalB against APAP-induced hepatotoxicity in vivo. As shown in Fig. 1A, APAP alone dramatically increased serum ALT and AST activity. However, when animals also received SalB pretreatment, the serum ALT and AST activity were significantly decreased compared to those of the APAP control. We further verified the protective effect of SalB by performing a histopathological evaluation of the liver. As shown in Fig. 1B, the vehicle produced no apparent abnormalities, whereas APAP-induced a disordered arrangement of hepatocytes,

Fig. 1. Effect of SalB on APAP-induced liver injury. Mice pretreated with SalB (25 or 50 mg/kg per day, i.g., for 3 days) received a single dose of APAP (300 mg/kg, i.g.). The animals were sacrificed 24 h after APAP administration. (A) The activity of ALT and AST from serum samples. (B) Hematoxylin and eosin staining of the liver (×400). (a) Mice treated with double-distilled water (DDS) were tested controls. (b) Mice treated with 50 mg/kg SalB. (c) APAP-induced liver injury. (d) SalB (50 mg/kg) pretreatment suppressed APAP-induced liver injury. Values are expressed as means ± SD, n = 10. **p < 0.01 vs. the control group; ##p < 0.01 vs. the APAP group.
3.2. SalB induces expression of Nrf2 and phase II enzyme genes

Cells can be protected from oxidative stress either by directly scavenging ROS (reactive oxygen species) or by fortifying the body’s antioxidant defenses. Therefore, we determined whether the protective effect of SalB against liver injury is associated with Nrf2 and phase II enzymes. In Fig. 2A, pretreatment of mice with SalB alone increased Nrf2, HO-1 and GCLC protein expression compared with that in vehicle controls. Moreover, pretreatment with SalB for 3 days before APAP administration significantly increased Nrf2, HO-1 and GCLC protein expression compared with that in mice receiving APAP. Additionally, we probed the effects of SalB on Nrf2, HO-1 and GCLC protein expression in vitro (Fig. 2B), which showed the same results as that in vivo. Consistent with protein expression, the corresponding mRNA expression in vivo and in vitro displayed the same trend (data not shown). To further investigate whether the protective effects of SalB are associated with induction of HO-1 and GCLC, we used specific HO-1 and GCLC inhibitors (ZnPP and BSO, respectively). The inhibitors abrogated the protective effect of SalB on HepG2 cells (Fig. 3). Taken together, these experiments suggested that SalB protects the liver from APAP-induced injury through induction of Nrf2, HO-1 and GCLC.

3.3. SalB-induced phase II enzyme expression is mediated by Nrf2

Given that most genes encoding phase II detoxifying and antioxidant enzymes contain an ARE, which has been reported as the mechanism through which Nrf2 regulates transcription (21), we next studied whether Nrf2 up-regulated the expression of HO-1 and GCLC. We examined HO-1 and GCLC expression after siRNA knockdown of Nrf2 in HepG2 cells. Silencing Nrf2 expression with specific siRNA abolished SalB-induced up-regulation of HO-1 and GCLC, whereas Nrf2 expression was not affected in the control siRNA group (Fig. 4). These results suggest that the SalB-induced expression of HO-1 and GCLC is regulated by the activation of Nrf2 signaling.

3.4. SalB enhances Nrf2-mediated phase II enzyme expression via activation of PI3K/AKT and PKC signaling

Several signaling pathways, including the MAPK, PI3K/AKT, and PKC pathways, are involved in the induction of Nrf2 activation (22,23). To elucidate the upstream signaling events leading to Nrf2

![Fig. 2](image-url). Effect of SalB on Nrf2, HO-1 and GCLC expression. (A) Effect of SalB on the protein levels of Nrf2, HO-1 and GCLC during APAP-induced liver injury in mice. Mice pretreated with SalB (25 or 50 mg/kg per day, i.g., for 3 days) received a single dose of APAP (300 mg/kg, i.g.). The animals were killed 24 h after APAP administration. (B) Effect of SalB on the protein levels of GCLC in HepG2 cells. Cells were pretreated with 0, 0.5, 2, or 8 μM SalB for 6 h. Values are expressed as means ± SD, n = 3. *p < 0.05 vs. the control group; **p < 0.01 vs. the APAP group; ***p < 0.01 vs. the SalB + APAP group.

![Fig. 3](image-url). Effect of selective HO-1 and GCLC inhibitors on HepG2 cell viability. HepG2 cells were pretreated with 20 μM ZnPP or 200 μM BSO for 2 h and then treated with 8 μM SalB for 6 h. Next, cells were further exposed to 10 mM APAP for 24 h to evaluate cell viability. *p < 0.01 vs. the control group; **p < 0.01 vs. the APAP group; ##p < 0.01 vs. the SalB + APAP group.
activation in SalB-treated cells, the following kinase inhibitors were used: GF109203X (for PKC), LY294002 (for PI3K), PD98059 (for extracellular signal-related protein kinases 1/2 (ERK1/2)), SB203580 (for P38), and SP600125 (for c-Jun N-terminal kinase (JNK)). The results indicated that inhibiting PI3K with LY294002 or the PKC pathways with GF109203X blocked the protective effect of SalB against cell death induced by APAP; however, the other inhibitors did not show this effect (Fig. 5A).

To further elucidate the roles of PKC and PI3K signaling in SalB-induced Nrf2 activation, we examined the effects of SalB on the activation of PKC and AKT, a PI3K downstream target. SalB significantly increased the protein phosphorylation levels of AKT and PKC, whereas no difference was found in the phosphorylation of ERK, P38 and JNK proteins (Fig. 5B). To further elucidate the molecular mechanism underlying Nrf2 activation, we attempted to determine the link between PI3K/AKT, PKC and Nrf2 activation induced by SalB. Interestingly, specifically inhibiting PI3K/AKT and PKC apparently abolished the ability of SalB to increase Nrf2, HO-1 and GCLC expression (Fig. 6A and B). These data indicated that PI3K/AKT and PKC are candidate kinases for the regulation of Nrf2, HO-1 and GCLC expression induced by SalB (Fig. 7).

4. Discussion

Radix Salvia miltiorrhiza is one of the most important traditional herbal medicines and is widely used to treat coronary artery disease, cardiovascular diseases and liver fibrosis [14,24]. SalB, the most abundant and bioactive ingredient of Radix Salvia miltiorrhiza, has raised considerable interest in recent years. Previous investigations have reported that SalB possesses hepatoprotective effects [15,25]. SalB suppresses TGF-β1 expression, decreases MAPK activity and effectively reverses the effects of liver fibrosis during chronic hepatitis B. In a study by Kong R et al, it was shown that SalB pretreatment protected liver tissue against ischemia-reperfusion injury through decreased post-ischemic oxidative stress, improved energy metabolism, and reduced hepatocellular dysfunction.

Fig. 4. Involvement of Nrf2 in SalB-induced HO-1 and GCLC expression. HepG2 cells were transfected with a Nrf2 siRNA or control siRNA for 6 h, followed by treatment with 8 μM SalB for an additional 42 h. Nrf2, HO-1 and GCLC were evaluated using Western blotting. Values are expressed as means ± SD, n = 3. ** p < 0.01 vs. the si-control group.

Fig. 5. Effect of selective kinase inhibitors on cell viability and protein expression in the presence of SalB. (A) Cell viability of HepG2 cells pretreated with 20 μM GF109203X, 20 μM LY294002, 20 μM PD98059, 20 μM SB203580 or 20 μM SP600125 for 1 h and then treated with 8 μM SalB for 6 h. The cells were then further exposed to 10 mM APAP for 24 h. Values are expressed as means ± SD, n = 10. ** p < 0.01 vs. the control group; ** p < 0.01 vs. the APAP group; && p < 0.01 vs. the SalB + APAP group. (B) Effect of SalB on the total and phosphorylation levels of AKT, PKC, ERK, P38, and JNK. Cells treated with 0, 0.5, 2, or 8 μM SalB for 6 h. Phosphorylated AKT, PKC, ERK, P38 and JNK were evaluated using Western blotting. Values are expressed as means ± SD, n = 3.
apoptosis (26). SalB was also reported to protect against platelet-derived growth factor (PDGF)-induced cell proliferation and migration, and these beneficial effects are associated with the induction of HO-1 via Nrf2 activation (27). In the present study, for the first time, we reported that SalB clearly protects hepatocytes against APAP-induced damage. The protective effect is mainly associated with up-regulation of HO-1 and GCLC expression through the activation of Nrf2 translocation and the stimulation of the PI3K/AKT and PKC pathways.

Numerous studies have shown that administering natural compounds to induce phase II enzymes is sufficient to promote antioxidant and protective activities. It is generally known that HO-1 and GCLC are principal phase II enzymes. HO-1 overexpression has been demonstrated to protect against ethanol-induced apoptosis (28). By contrast, suppression of HO-1 activity results in increased hepatocellular injury, apoptosis, and death from infection/sepsis in mice (29). In addition, certain HO-1 inducers have the potential to protect hepatocytes against oxidative injury (30). GCLC is the rate-limiting enzyme in the synthesis of glutathione, a major antioxidant molecule in cells. Previous studies have shown that oleanolic acid lessens APAP-induced cellular damage by up-regulating GCLC expression (31). Consistent with these observations, in the present study, we found that SalB-induced HO-1 and GCLC expression in liver tissue and HepG2 cells (Fig. 2). More importantly, the protective effect of SalB against APAP injury in HepG2 cells was attenuated by the HO-1 inhibitor ZnPP and the GCLC inhibitor BSO, suggesting that the former effect is associated with HO-1 and GCLC (Fig. 3).

Fig. 6. The roles of PKC and PI3K signaling in SalB-induced Nrf2, HO-1 and GCLC expression. HepG2 cells were pretreated with 20 μM GF109203X or 20 μM LY294002 for 1 h and then treated with 8 μM SalB for 6 h. AKT, PKC, Nrf2, HO-1 and GCLC levels were assessed using Western blotting. PKC, Nrf2, HO-1 and GCLC levels were assessed using Western blotting. Values are expressed as means ± SD, n = 3. **p < 0.01 vs. the control group.

Fig. 7. Scheme summarizing the inhibition of APAP-induced liver injury by SalB via the up-regulation of Nrf2, HO-1 and GCLC expression through the PI3K and PKC pathways.
regulated Nrf2 expression in vivo and in vitro. To determine the involvement of Nrf2 in SalB-induced HO-1 and GCLC expression, we used RNA interference directed against Nrf2. The results indicated that silencing Nrf2 with siRNA abolished SalB-induced Nrf2, HO-1 and GCLC protein expression (Fig. 4), suggesting that the SalB-induced up-regulation of HO-1 and GCLC depends on increased nuclear expression of Nrf2.

It has been reported that mice administration of APAP could increase cytoprotective genes, including Nrf2, NAPDH quinone oxidoreductase 1 (Nqo1) and GCLC, which may provide insight into adaptive recovery mechanisms and development of resistance of cytotoxic xenobiotics (34). Combined with the previous results, we also found that APAP itself increased Nrf2, HO-1 and GCLC protein expression, which suggested that cellular defense is able to act in concert to resist harmful reactive intermediates formed from APAP in a certain extent. In other words, increased Nrf2, HO-1 and GCLC expression is an adaptive response to APAP-induced hepatitis.

Several cytosolic kinases, such as MAPK, PI3K/AKT and PKC, have been shown to modulate Nrf2 and participate in signal transduction from antioxidants and xenobiotics to ARE (35,36). ERK, JNK, and p38 are the primary members of the MAPK family. We therefore observed the effects of SalB on the expression levels of MAPKs, PI3K/AKT and PKC. Our results suggested that AKT and PKC, but not p38, JNK or ERK are mainly activated in the presence of SalB (Fig. 5B). Our observations therefore support the hypothesis that PI3K/AKT and PKC play an important role in the protective effect of SalB against liver injury induced by APAP overdose. In the present study, we demonstrated that the PI3K/AKT inhibitor LY2943902 and the PKC inhibitor GF109203X alleviated the cytoprotective effect of SalB against APAP-induced cell death (Fig. 5A). Consequently, we concluded that SalB triggers PI3K and PKC signaling to activate the Nrf2 signaling pathway, a process that is, at least in part, responsible for the protective effect against APAP-induced hepatotoxicity.

In summary, we demonstrated for the first time that SalB protects the liver from APAP-induced injury by up-regulating HO-1 and GCLC expression via the activation of Nrf2 nuclear translocation and that the PI3K/AKT and PKC pathways are involved in this process. These findings provide a rationale for potential clinical applications of SalB for the prevention or treatment of liver intoxication.

Conflict of interest
There are no conflicts of interest to disclose for any of the authors.

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