Antroquinonol from ethanolic extract of mycelium of Antrodia cinnamomea protects hepatic cells from ethanol-induced oxidative stress through Nrf-2 activation

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Aim of the study: In recent years, the medicinal mushroom Antrodia cinnamomea, known as “niu-chang chih“ has received much attention with regard to its possible health benefits; especially its hepatoprotective effects against various drugs, toxins, and alcohol induced liver diseases. However, the molecular mechanism underlying this protective effect of Antrodia cinnamomea and its active compound antroquinonol was poorly understood. In the present study we evaluated to understand the hepatoprotective efficacy of antroquinonol and ethanolic extracts of mycelia of Antrodia cinnamomea (EMAC) in vitro and in vivo.

Materials and methods: The protective mechanism of antroquinonol and EMAC against ethanol-induced oxidative stress was investigated in cultured human hepatoma HepG2 cells and ICR mice model, respectively. HepG2 cells were pretreated with antroquinonol (1–20 \( \mu \)M) and oxidative stress was induced by ethanol (100 mM). Meanwhile, male ICR mice were pretreated with EMAC for 10 days and hepatotoxicity was generated by the addition of ethanol (5 g/kg). Hepatic enzymes, cytokines and chemokines were determined using commercially available assay kits. Western blotting and real-time PCR were subjected to analyze HO-1 and Nrf-2 expression. EMSA was performed to monitor Nrf-2 ARE binding activity. Possible changes in hepatic lesion were observed using histopathological analysis.

Results: Antroquinonol pretreatment significantly inhibited ethanol-induced AST, ALT, ROS, NO, MDA production and GSH depletion in HepG2 cells. Western blot and RT-PCR analysis showed that antroquinonol enhanced Nrf-2 activation and its downstream antioxidant gene HO-1 via MAPK pathway. This mechanism was then confirmed in vivo in an acute ethanol intoxicated mouse model: serum ALT and AST production, hepatocellular lipid peroxidation and GSH depletion was prevented by EMAC in a dose-dependent manner. EMAC significantly enhanced HO-1 and Nrf-2 activation via MAPKs consistent with in vitro studies. Ethanol-induced hepatic swelling and hydropic degeneration of hepatocytes was significantly inhibited by EMAC in a dose-dependent manner.

Conclusions: These results provide a scientific basis for the hepatoprotective effects of Antrodia cinnamomea. Data also imply that antroquinonol, a potent bioactive compound may be responsible for the hepatoprotective activity of Antrodia cinnamomea. Moreover, the present study highly supported our traditional knowledge that Antrodia cinnamomea as a potential candidate for the treatment of alcoholic liver diseases.

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1. Introduction

Alcohol-induced liver diseases usually occur after years of excessive alcohol consumption. The longer the period of time alcohol is consumed and the more alcohol that is ingested, the greater the likelihood of developing liver diseases (Younossi, 1998). Acute alcoholic hepatitis can also result from binge drinking and is life-threatening if severe (De et al., 2009). Mounting scientific literature indicates that excess alcohol ingestion increases hepatic enzymes, cytokines and reactive oxygen species such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor-alpha (TNF-α), reactive oxygen species (ROS), nitric oxide (NO) and cellular lipid peroxidation, which play a major role in the pathogenesis and progression of alcohol-induced liver diseases (Guitierrez-Ruiz et al., 1999; Nah et al., 2005). In addition, alcohol consumption leads to excessive generation of free radicals that result in spontaneous depletion of hepatic glutathione (GSH) levels (Guitierrez-Ruiz et al., 1999).

NF-E2 related factor-2 (Nrf-2) is a transcription factor known to induce expression of a variety of cytoprotective and detoxifying genes (phase II genes) through a promoter sequence named the antioxidant responsible element (ARE) (Yang et al., 2009). In unstressed conditions Nrf-2 is tethered in the cytoplasm to kelch-like ECH associated protein-1 (Keap-1), thus preventing its nuclear translocation and acting as a transcriptional repressor. Under conditions of stress, Nrf-2 is released from Keap-1 repression and translocates into the nucleus where it transcribes a number of antioxidant and/or detoxification genes such as heme oxygenase (HO-1), quinone oxidoreductase (NQO1), glutathione S-transferase A2 (GSTA-2) and the two subunits of heme oxygenase (HO-1), quinone oxidoreductase (NQO1), glutathione antioxidant and/or detoxification genes such as heme oxygenase (HO-1), quinone oxidoreductase (NQO1), glutathione S-transferase A2 (GSTA-2) and the two subunits of γ-glutamylcysteine ligase (γ-GCL, γ-GCLC and γ-GCLM) (Surh, 2003). HO-1 is a rate limiting enzyme that catalyses heme into carbon monoxide, biliverdin and ferrous iron. Biliverdin is then further converted into bilirubin, a potent endogenous antioxidant (Bao et al., 2010). Increasing evidence suggests that HO-1 plays a central role in protecting the liver against alcohol-induced hepatotoxicity (Farombi and Surh, 2006).

Alcohol-induced elevation of ALT, AST, lipid peroxidation and glutathione depletion has been well documented in both hepatic cell lines and rodent models (Guitierrez-Ruiz et al., 1999; Kanbak et al., 2001; Nah et al., 2005). Previous studies strongly suggest that enhancement of cellular detoxifying enzymes or inhibition of hepatic enzymes can down-regulate development and pathogenesis of alcohol-induced liver diseases (Das and Vasudevan, 2007). Antrodia cinnamomea is a rare and very precious medicinal mushroom in Taiwan. Antrodia cinnamomea is known as “niu-chang-chih” in Chinese and has been referred to as a “national treasure of Taiwan” (Shen et al., 2003). This medicinal mushroom was historically used only by native Taiwanese as a traditional prescription for the discomforts caused by alcohol drinking or exhaustion because of their life style (Su, 2002). In the year of 1773, a famous traditional Chinese medicine practitioner known him Dr. Wu-Sha, found that Taiwan aborigines had discomfort caused by excesses alcohol or exhaustion. However, the locals often chewed raw fruiting bodies or decoction of fruiting bodies of Antrodia cinnamomea to prevent alcohol hangover (Ao et al., 2009). In addition, the fruiting bodies of Antrodia cinnamomea also has been used as a Chinese folk medicine for the treatment of liver diseases, food and drug intoxication, diarrhea, abdominal pain, hypertension, allergies, skin itching and tumorigenic diseases (Huang et al., 2006; Ao et al., 2009). After being used for years as a traditional medicine, this mushroom now believed to be one of the most potent liver protecting herb in Taiwan. Recent scientific literature has also shown that this mushroom possesses a number of bioactive properties such as anti-cancer, anti-inflammatory, anti-oxidant, anti-hypertensive, anti-hepatitis B virus replication, hepatoprotective and neuroprotective functions (Ao et al., 2009; Geethangili and Tzeng, 2009). This fungus has even been suggested for the treatment of diabetes and cardiovascular diseases (Su, 2002; Wang et al., 2003). Antrodia cinnamomea is rich in flavonoids, terpenoids, polyphenolics and polysaccharides (Chiang et al., 2010). Antroquinonol, a ubiquinone derivative isolated from the fruiting bodies and mycelium of Antrodia cinnamomea, showed interesting bioactive properties such as anti-cancer and anti-inflammatory activity (Lee et al., 2007; Chang et al., 2008; Yang et al., 2009; Chiang et al., 2010). It has been reported that mycelia of Antrodia cinnamomea possess hepatoprotective effects against ethanol-induced liver injury in rats (Lu et al., 2007). However, the molecular mechanism underlying this hepatoprotective drug is poorly understood. In the present study we revealed the molecular mechanism of the hepatoprotective function of ethanolic extract from mycelium of Antrodia cinnamomea (EMAC) and its pure derived compound antroquinonol in vitro and in an acute ethanol intoxicated mouse model.

2. Materials and methods

2.1. Chemicals and reagents

Antrodia cinnamomea mycelia were obtained from R&D Center of Taiwan Leader Biotechnology Corporation (Taichung, Taiwan). To prepare crude ethanolic extract of mycelia of Antrodia cinnamomea, fresh air dried mycelia were immersed in ethanol for a week. Extracts were filtered through Whatman No. 1 filter paper and then ethanol was removed by rotary evaporator. Antroquinonol (Fig. 1) was purified from mycelia of Antrodia cinnamomea according to a protocol described previously (Lee et al., 2007). The purity of antroquinonol was above 99% according to HPLC and 1H NMR analysis. Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Gries reagent, silymarin, 2,7-dichlorofluorescein diacetate (DCF-DA), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide ( MITT) and mouse monoclonal anti-β-actin antibody were purchased from Sigma–Aldrich (ST. Louis, CA, USA). Mouse monoclonal HO-1 and rabbit polyclonal Nrf-2 antibodies were obtained from Abcam (Cambridge, MA, USA). Cell culture dishes and multi-well plates were purchased from Techno Plastic Products (Trasadingen, Switzerland).

2.2. Cell culture and sample treatment

Human hepatoma (HepG2) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in MEM supplemented with FBS, glucose, glutamine, penicillin and streptomycin. This cell line has been utilized in various ethanol-induced hepatotoxicity studies (Guitierrez-Ruiz et al., 1999; Nah et al., 2005; Kaur et al., 2009). HepG2 cells were grown in –10 cm2 cell culture dish and incubated in a humidified atmosphere containing 5% CO2 in air at 37°C. Cultures were allowed to reach 80–90% confluence before experiments were initiated. Cells (1 × 10^2 cells/mL) were incubated in the presence or absence of...
different concentrations of antroquinonol (5, 10 and 20 \( \mu \)M) or silymarin (100 \( \mu \)M) and then hepatotoxicity was induced by addition of ethanol (100 mM) in to the culture medium. The biological relevance of this ethanol concentration was supported by previous studies (Guitierrez-Ruiz et al., 1999; Kaur et al., 2009). Cells received 1% of DMSO instead of sample or ethanol. Commercially available silymarin was used as a positive control for all subsequent studies.

2.3. Cell viability assay

Cell viability was assessed by MTT assay as described previously (Nah et al., 2005). In brief, HepG2 cells were plated in 96-well microtiter plate at a density of 1 \( \times \) 10\(^4\) cells/mL in a final volume of 200 \( \mu \)L culture medium per well and left for 2 h. Cells were further incubated in the presence or absence of various concentrations of antroquinonol and/or ethanol for 24 h. After incubation for 24 h the medium was removed and MTT (10 \( \mu \)g/mL) was mixed with 90 \( \mu \)L culture medium to allow intracellular reduction of soluble yellow MTT into insoluble purple formazan crystal. Supernatant was discarded and the remaining MTT crystals were dissolved in 100 \( \mu \)L of DMSO. The optical absorbance of samples was measured at 570 nm using enzyme-linked immunosorbent assay (ELISA) microplate reader (\( \mu \)Quant, Bio-Tek Instruments, Winooski, VT, USA) and percentage viability was calculated.

2.4. Determination of hepatic ALT and AST production

The levels of ALT and AST in the culture medium were measured using commercially available assay kits (Randox Laboratories, Ardrin, UK). Briefly, HepG2 cells were incubated in the presence or absence of various concentrations antroquinonol for 1 h and then the cells were stimulated with ETOH for 24 h. After incubation, culture supernatant was removed and total ALT and AST were measured at 340 nm using an ELISA microplate reader. Results were expressed as unit per liter.

2.5. Determination of lipid peroxidation and glutathione

Ethanol-derived hepatic GSH and lipid peroxidation were assayed in HepG2 cells. Briefly, 1 \( \times \) 10\(^4\) cells/mL were seeded in 6-well culture plates. Cells were pretreated with various concentrations of antroquinol or silymarin for 1 h. Toxicity was induced by the addition of ethanol to the culture medium for 24 h. Cells were lysed with 100 \( \mu \)L of lysis buffer. Lysates were centrifuged at 12,000 \( \times \) g for 15 min at 4 \( ^\circ \)C, and the supernatant was mixed with 100 \( \mu \)L trichloroacetic acid (as a precipitation reagent, provided in commercially available GSH assay kit (Oxis International, Foster City, CA, USA). Meanwhile, lipid peroxidation was determined by the formation of MDA in cultured cell lysates by using a lipid peroxidation assay kit (Oxford Biomedical Research, Oxford, MI, USA) following the supplier’s instructions.

2.6. Determination of intracellular ROS accumulation

Intracellular ROS accumulation in HepG2 cells was monitored using fluorescent marker DCF-DA as described previously (Nah et al., 2005) with minor modifications. DCF-DA is a nonfluorescent compound that is deacetylated by viable cells to 2′,7′-dichlorofluorescein (DCF) by ROS. HepG2 cells (1 \( \times \) 10\(^7\) cells/mL) were seeded in 24-well plates and treated with or without various concentrations of antroquinol or silymarin for 1 h. Oxidative stress was induced by the addition of ethanol to the culture medium for 24 h. At the end of the incubation period, the culture supernatant was removed and cells were washed twice with PBS. DCF-DA (10 \( \mu \)M) was mixed with Kreb’s buffer and added to the culture plate. After incubation for 30 min, relative levels of fluorescence were quantified using a fluorescence spectrophotometer (Chemeleon V, Hiexid Oy, Turku, Finland) at 485/535 nm.

2.7. Protein extraction and western blot analysis

Cytoplasmic and nuclear fractions were obtained by using commercially available nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL, USA). HepG2 cells (1 \( \times \) 10\(^7\) cells/mL) were cultured in 6-cm dishes and incubated with various concentration of antroquinol or silymarin for 1 h. Oxidative stress was then induced by the addition ethanol to the culture medium for 2–24 h. At the end of incubation, cells were lysed with cytoplasmic and nuclear extraction reagent. The protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). For western blot analysis, 20–50 \( \mu \)g protein fractions were separated by 7–12% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane. Western blots were performed with appropriate antibodies using the ECL western blotting reagent (Millipore, Billerica, MA, USA) and the image was visualized by a Vil Chemi-Smart 3000 (Viogene Biotek, Sunnyvale, CA, USA) imaging device.

2.8. Electrophoretic mobility shift assay

EMSA was performed to determine Nrf-2 ARE binding activity using a commercially available fluorescence-based EMSA kit (Invitrogen, Carlsbad, CA, USA). In brief, oligonucleotide probes: hARE (human anti-oxidant responsive element), forward primer 5′-CTC GAG CCC TAT AAC TGC TAT CTC-3′, reverse primer 5′-AAG CCT GTC TCT GGT GCA GTC CGG-3′ were synthesized by SBS Biotech (Taipei, Taiwan), annealed with TE buffer for 5 min at 94 °C and then gradually cooled for 3 h. Nuclear extract (20 \( \mu \)g) was incubated with 20 ng of double-standard hARE oligonucleotides for 30 min at room temperature along with binding buffer. The DNA protein complex was separated by 6% native polyacrylamide gel. The complex was then visualized using fluorescence-based EMSA reagent, and lumenescence intensity was quantitated using a Li Chemi-Smart 3000 (Vilber Lourmat, Torcy Z.I. Sud, France) imaging device.

2.9. RNA extraction and quantitative real-time PCR

Total RNA extraction and RT-PCR was performed as described previously (Kumar and Wang, 2009). In brief, total RNA was extracted from cultured HepG2 cells using the Trizol Reagent method. Real-time-PCR analysis was performed using Applied Biosystems detection instruments and software. Forward and reverse primers (10 mM), and the working solution SYBR green, used as a PCR master mix, were used under the following conditions: 96 °C for 3 min followed by 40 cycles at 96 °C for 1 min, 50 °C for 30 s and 72 °C for 90 s. β-Actin, a housekeeping gene, was chosen as an internal standard to control for variability in amplification because of differences in starting mRNA concentrations. The sequences of the PCR primers were as follows: HO-1, forward 5′-TGC GGT GCA GCT CTT CTG-3′, reverse 5′-GCA ACC CAG CAT GC-3′; β-actin, forward 5′-ACC CAC ACT GTG CCC ATC TA-3′; reverse 5′-CCG AAC CGC TCA TTT GC-3′. The copy number of each transcript was calculated as the relative copy number normalized by β-actin copy number.

2.10. Animals and experimental protocols

Ethanol-induced acute hepatotoxicity studies were carried out as per the binge drinking method described previously.
Fig. 2. Effects of antroquinonol on ethanol-induced ALT, AST, MDA and GSH depletion in HepG2 cells. Cells were pre-incubated with indicated concentrations of antroquinonol and silymarin for 1 h and stimulated by ethanol for 24 h. Hepatic ALT (A), AST (B) was measured in culture supernatant. Hepatocellular MDA (C) and GSH depletion (D) was assessed from HepG2 cell lysates. Values represent the mean ± SD of three experiments. *p < 0.05, **p < 0.01, ***p < 0.001 was considered significant for EtOH alone. †p < 0.05 was considered significant for control vs EtOH alone.

and Pruett, 1996) with minor modifications. Four-week-old male ICR mice purchased from Charles River (Taipei, Taiwan) weighing 25 ± 5 g were used for this study. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and Taiwan laws relating to the protection of animals, and were approved by the local ethics committee. Thirty male ICR mice were divided into five mice in each group. Mice from groups I and II served as the control and received vehicle (2% DMSO in ddH2O) throughout the experiment. Groups III–V received 250, 500 and 1000 mg/kg of EMAC, respectively, once a day for 10 days (Dai et al., 2003). Silymarin was administered to group VI at a dose of 200 mg/kg for 10 days via oral gavage (Song et al., 2006). At the end of drug treatment, hepatotoxicity was induced by administering ethanol (5 g/kg of body weight) to groups II–VI via oral gavage in three doses at 12 h intervals. Mice were sacrificed by anesthesia with ethyl ether. Blood was collected in EDTA tubes and centrifuged at 15,000 × g for 10 min at 4 °C. Serum was obtained and stored at −20 °C for further examination. Liver from one mouse in each group was removed, and kept in −80 °C for further cellular analysis. Meanwhile, remaining livers was washed with PBS and kept in 10% neutral buffered formalin (NBF) for further histopathological analysis.

2.11. Determination of serum ALT, AST, GSH and lipid peroxidation

ALT and AST in serum were monitored using Randox clinical test kits. Total cellular GSH and lipid peroxidation was measured in non protein liver homogenates. Briefly, 1 g of liver section was used to prepare homogenate in 500 μL of PBS and 0.5% Triton X-100 solution. The homogenate were centrifuged in 16,000 × g for 10 min at 4 °C. The cellular GSH level of the resulting supernatant was assessed by using reduced GSH assay kit. Malondialdehyde (MDA), a byproduct of lipid peroxidation, was assayed in liver homogenates by commercially available lipid peroxidation assay kit.

2.12. Tissue protein preparation and western blot analysis

Preparation of cytosolic and nuclear protein fraction was described previously (Kumar et al., 2010). Briefly, 1 g of liver section was homogenized in liquid nitrogen using a pestle and morter. Homogenates (50 mg) were centrifuged at 500 × g for 5 min at 4 °C. Cytosolic and nuclear fractions were obtained using commercially available nuclear and cytoplasmic extraction reagent kits. Protein quantification and western blot analysis was as described above.

2.13. Histopathological study

Liver sections were then analyzed by a veterinary pathologist for evidence of hepatic swelling and hydropic degeneration of hepatocytes following ethanol administration compared to control group. Livers of EMAC pre-treatment groups and control groups were compared for protective effect by means of possible differences in liver histology and hepatic lesions induced by ethanol. Fresh liver tissues were trimmed to a thickness of 3 mm, placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of ethanol-induced necrosis was evaluated by assessing the morphological changes in liver sections stained with hematoxylin and eosin.

2.14. Statistical analysis

Data are expressed means ± SD. Statistical analysis of the results were made using Dunnett’s test for multiple comparison and
3. Results

3.1. Cytotoxic effects of antroquinonol on human hepatoma HepG2 cells

Prior to in vitro hepatoprotective studies, the cytotoxic effect of antroquinonol was examined in human hepatoma HepG2 cells. Cells (1 × 10⁷ cells/mL) were incubated in various concentrations of antroquinonol (5, 10, 20, 40 and 80 μM) for 24 h. In MTT assay, cell survival was more than 90% up to a concentration of 20 μM (Fig. 1SA). At concentrations of 40 and 80 μM of antroquinonol, cell survival was reduced to 60% and 40% respectively. In addition, we also observed that antroquinonol at concentrations of 5, 10 and 20 μM had almost no affect on cell viability when co-treated with 100 mM ethanol (Fig. 1SB).

3.2. Antroquinonol reduces hepatic enzyme levels in ethanol-induced HepG2 cells

Hepatic transaminase levels were measured after ethanol induction in HepG2 cells. After exposure to ethanol for 24 h, ALT and AST levels were markedly increased in culture media. Pretreatment with antroquinonol, significantly reduced ethanol-induced elevation of ALT and AST in a dose-dependent manner (Fig. 2A and B). Of note, ethanol-induced hepatic ALT and AST levels were also decreased by the positive control silymarin (Fig. 2A and B). To examine the effect of antroquinonol on ethanol-induced lipid peroxidation, the level of MDA in culture lysates was measured. Ethanol treatment increased intracellular MDA levels in HepG2

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cells remarkably (Fig. 2C). When cells were pretreated with antroquinonol, MDA elevation induced by ethanol was significantly reduced in a dose-dependent manner (Fig. 2C). GSH is the most abundant endogenous antioxidant in hepatocytes. During oxidative stress, increase in GSH consumption results in GSH depletion at the cellular level. Cultured HepG2 cells treated with ethanol had reduced GSH levels. However, pretreatment with antroquinonol protected against hepatic GSH depletion, as evidenced by the restoration or accumulation of GSH above normal levels (Fig. 2D). Pretreatment with the positive control silymarin also caused highly significant inhibition of MDA and restoration of GSH levels in ethanol-induced HepG2 cells in comparison with cells exposed to ethanol alone (Fig. 2C and D).

3.3. Antroquinonol suppresses ethanol-induced ROS generation and decreases NO production in HepG2 cells

In order to verify the antioxidant properties of antroquinonol, ethanol-induced oxidative stress in HepG2 cells was directly measured by intracellular ROS accumulation using DCF-DA fluorescence. Exposure of cells to ethanol for 24 h increased intracellular ROS levels in comparison with control cells (Fig. 3A); however, pretreatment with antroquinonol significantly suppressed ethanol-induced intracellular ROS accumulation in a dose-dependent manner. In contrast, silymarin failed to prevent ROS generation in cultured HepG2 cells (Fig. 3A). Furthermore, prevention of ROS accumulation by antroquinonol pretreatment was able to decrease production of NO in ethanol-induced HepG2 cells, as demonstrated by measurement of the accumulation of nitrite in the culture supernatant (Fig. 3B).

3.4. Antroquinonol increases HO-1 mRNA expression and protein levels in ethanol-induced HepG2 cells

It is well known that HO-1 is a major antioxidant enzyme that plays an important role in ethanol-induced antioxidant defense in hepatic cells (Bao et al., 2010). We hypothesized that the inhibitory effects of antroquinonol on ethanol-induced hepatic enzyme leakage and/or augmentation of GSH level result from the induction of antioxidant genes such as HO-1 and NQO1. As expected, we observed antroquinonol significantly increased HO-1 mRNA expression levels in ethanol-induced HepG2 cells (Fig. 4A). This was confirmed by western blot, which showed that antroquinonol augmented HO-1 protein level in a dose-dependent manner (Fig. 4B). Silymarin at a dose of 100 μM also enhanced both HO-1 mRNA and protein expression levels. In addition, antroquinonol and silymarin significantly inhibited ethanol-induced TNF-α level in HepG2 cells (Fig. 4B). In contrast, antroquinonol and silymarin did not show any significant effect on NQO1 mRNA or protein expression in ethanol challenged HepG2 cells (data not shown).

3.5. Antroquinonol increases Nrf-2 activation and Nrf-2 ARE binding activity in ethanol-induced HepG2 cells

Recent studies demonstrated that HO-1 can be activated by Nrf-2, a major transcription factor regulating ARE-driven phase-II gene expression (Surh, 2003). We, therefore, attempted to determine whether antroquinonol could activate Nrf-2 in association with its HO-1 up-regulation in cultured HepG2 cells. Moreover, it is well known that during stimulation, Nrf-2 dissociates from Keap-1 and translocates into the nucleus. Activation of Nrf-2 was determined by western blot using nuclear extracts from cultured HepG2 cells. As illustrated in Fig. 5A, antroquinonol treatment increased Nrf-2 accumulation in the nuclear fraction; however, the amount of cytosolic Nrf-2 was not affected by antroquinonol. To understand the potential role of antroquinonol in Nrf-2 transcriptional activation, EMSA was performed using the oligonucleotide harboring HO-1 specific ARE sequence. As shown in Fig. 5B, antroquinonol significantly increased Nrf-2 ARE binding activity in a dose-dependent manner. Silymarin showed more Nrf-2 translocation and ARE DNA binding activity than antroquinonol (Fig. 5A and B).

3.6. Effect of antroquinonol on MAPK activation in HepG2 cells

Activation of mitogen-activated protein kinases (MAPKs) can result in translocation of Nrf-2 leading to ARE-driven antioxidant gene expression (Surh, 2003). Bear this in mind; we further investigated the potential effect of antroquinonol on activation of MAPK family proteins such as p38, extracellular signal-regulated protein kinases (ERKs) and c-Jun N-terminal kinases (JNKs) in HepG2 cells. As shown in Fig. 6, antroquinonol significantly activated p38MAPK and JNK/SAPK protein phosphorylation in a dose-dependent manner. However, we found that antroquinonol failed to activate ERK protein phosphorylation (Fig. 6).

3.7. Effect of EMAC on ethanol-induced hepatotoxicity in mice

The effects of EMAC pretreatment on ethanol-induced elevation of serum ALT, AST production are shown in Fig. 7. In comparison with the control group, acute ethanol administration resulted in severe hepatic injury as shown by dramatic increases in serum hepatic enzyme levels such as ALT and AST (Fig. 7A and B). However, pretreatment with EMAC significantly reduced ethanol-induced hepatic enzyme levels in a dose-dependent manner (Fig. 7A and B).

We also observed that ethanol treatment significantly decreased hepatic GSH levels in comparison with the non-stimulated control group (Fig. 7C). EMAC pretreatment significantly protected the liver against ethanol-induced GSH consumption. In order to evaluate the effect of EMAC on ethanol-induced hepatic lipid peroxidation, we determined the MDA levels in liver homogenates. As shown in Fig. 7D, when compared to the non ethanol-stimulated control group, MDA production was severely increased in the ethanol-challenged group. Mice treated with EMAC significantly decreased ethanol-induced MDA elevation in hepatic tissues (Fig. 7D). Silymarin, at a dose of 200 mg/kg, also significantly protected mice against ethanol-induced hepatic enzyme elevation, lipid peroxi-
Fig. 7. Effects of EMAC on ethanol-induced serum ALT, AST elevation and hepatocellular MDA and GSH depletion in mouse liver tissues. Mice were pretreated with indicated concentrations of EMAC and silymarin for 10 days and hepatotoxicity was stimulated by ethanol for 36 h. Mouse blood serum ALT (A) and AST (B) were measured. Hepatocellular GSH depletion (C) and MDA (D) were assessed from mice liver lysates. Values represent the mean ± SD of three experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ were considered significant for EtOH alone. $\dagger p < 0.05$ was considered significant for control vs. EtOH alone.

dation and GSH depletion. Similar results were also observed for silymarin in previous studies (Song et al., 2006).

3.8. Effect of EMAC on ethanol-induced HO-1 and Nrf-2 augmentation in mice liver tissues

Our preliminary studies showed that EMAC pretreatment resulted in a dose-dependent protective effect against ethanol-induced hepatic enzyme release. Therefore, we assessed whether this protective function was due to activation of antioxidant genes. The cytosolic fraction was prepared from ethanol-induced mice liver tissues and analyzed by western blot. Pretreatment with EMAC increased HO-1 protein expression level in dose-dependent manner (Fig. 8A). Nrf-2 translocation is necessary for HO-1 activation (Surh, 2003). We thus monitored the Nrf-2 nuclear translocation and DNA binding ability of EMAC. Nrf-2 accumulation correlated with dose of EMAC in the nuclear fraction of mouse liver tissues (Fig. 8A). To elucidate the role of Nrf-2 in transcriptional activation of ARE, EMSA was performed. EMAC pretreated mice showed an increase in ARE DNA complex formation in a dose-dependent manner (Fig. 8B). Induction of Nrf-2 nuclear translocation and ARE DNA binding complex was also observed in ethanol-induced mouse liver tissues pretreated with silymarin (Fig. 8). Moreover, we also observed EMAC-treatment significantly induced phosphorylation of p38, JNK/SAPK and p42/p44 proteins in mice liver tissues (Fig. 9). These data was highly correlated with antroquinonol induced phosphorylation of MAPKs in cultured human hepatoma HepG2 cells (Fig. 6).

3.9. EMAC prevents acute ethanol-induced liver injury

Acute ethanol-induced liver injury is indicated by the leakage of hepatic enzymes into circulating blood. This is considered...
Fig. 9. Effects of EMAC on ethanol-induced MAPK activation in mouse liver lysates. Cyclophilin lysates were prepared and subjected to western blot to analyze phosphorylated and non-phosphorylated forms of p38, JNK/SAPK and p42/p44 ERK protein expression levels. One of three independent experiments is shown.

as a first sign of alcohol-induced liver damage, and progression is characterized by swelling and hydropic degeneration of hepatocytes (Zhao et al., 2008). Mice challenged with ethanol alone (5 g/kg) showed severe histopathological changes such as swelling and hydropic degeneration of hepatocytes (Fig. 10B). However, pretreatment with EMAC significantly prevented the above mentioned histopathological changes in a dose-dependent manner (Fig. 10C–E). The positive control, silymarin, a well-known hepatoprotective drug, showed highly significant protective effect against ethanol-induced hepatic swelling and hydropic (Fig. 10F). In contrast, the normal control group showed moderate histopathological changes in mouse liver tissues (Fig. 10A).

4. Discussion

In humans, acute or chronic alcohol ingestion results in alcoholic liver diseases that are difficult to manage with conventional medical therapies due to adverse side effects. Naturally derived substances are thus an attractive avenue in the search for suitable therapies. Several compounds from the mycelia of Antrodia cinnamomea (EMAC) have been reported to possess biological activities (Ao et al., 2009). Antroquinonol, a ubiquinone derivative isolated from the mycelia of Antrodia cinnamomea showed potent anti-cancer activity against various human carcinoma cell lines (Lee et al., 2007; Chiang et al., 2010; Yang et al., 2009) also described the anti-inflammatory activity of antroquinonol against lipopolysaccharide-induced inflammation in murine macrophage cells. In addition, preclinical toxicology studies of antroquinonol showed that toxic effects were observed only after administration of 30–50 times the effective dosage (Liu et al., 2008).

In the present study, human hepatoma HepG2 cells treated with ethanol increased levels of hepatic enzymes such as ALT and AST showed leakage from the cytoplasm into the culture medium through lipid peroxidation and membrane damage. Ethanol treatment also led to sustained depletion of GSH in HepG2 cells in agreement with previous studies (Gutierrez-Ruiz et al., 1999; Kaur et al., 2009). Antroquinonol pretreatment protected HepG2 cells against ethanol induced hepatic enzyme generation as well as cellular lipid peroxidation in a dose-dependent manner (Fig. 2). Antroquinonol pretreatment also reversed sustained GSH depletion as a result of ethanol exposure, which also supports previous observations (Kaur et al., 2009).

Ethanol exposure results in oxidative stress and elevated intracellular ROS accumulation in hepatic cells and these changes play a key role in the development and pathogenesis of alcoholic liver diseases (Das and Vasudevan, 2007). Ogny et al. (2008) demonstrated that incubation of HepG2 cells with ethanol induces oxidative stress and leaves the cells vulnerable to further injury by ROS. Ethanol exposure also increased intracellular ROS accumulation in HepG2 cells in this study; however, pretreatment with antroquinonol or silymarin inhibited this ethanol-induced ROS generation. Nitric oxide (NO) contributes to liver damage in several inflammatory liver diseases (Majano et al., 2004). This study also showed that induction of alcohol in HepG2 cells increased NO production; however, antroquinonol pretreatment was able to decreased this ethanol-induced NO production significantly.

HO-1 is a microsomal enzyme that catalyzes the oxidation of heme into antioxidant molecules, biliverdin and carbon monoxide. HO-1 has been shown to have a protective effect in several disparate models of hepatic injury (Farombi and Surh, 2006). Induction of HO-1 is generally recognized as an important therapeutic target for pharmacological intervention of oxidative disorders (Qi et al., 2010). Bao et al. (2010) showed that ethanol induction markedly decreased endogenous HO-1 activity in human hepatocytes. The current study suggests that ethanol exposure slightly attenuates HO-1 expression in control cells. This attenuation was significantly increased by antroquinonol treatment in a dose-dependent manner (Fig. 4). Nrf-2 is a basic leucine zipper transcription factor that binds and activates the antioxidant responsive element (ARE) in the promoter regions of many antioxidant genes including HO-1, NQO-1, GSTA-2, γ-GCLC and γ-GCLM (Surh, 2003). During oxidative stress, Nrf-2 dissociates from Keap-1 and translocates into the nucleus where it binds and activates antioxidant genes. We hypothesized that the increased level of HO-1 gene and protein expression could be due to activation of Nrf-2 signaling pathway. As we expected, antroquinonol increased Nrf-2 translocation and activation of the antioxidant responsive element in ethanol-induced HepG2 cells (Fig. 5). These results agree with other’s observation in different compounds (Yao et al., 2007; Bao et al., 2010; Qi et al., 2010).

On the basis of these in vitro findings, we performed further studies in vivo studies using ethanolic extract of the mycelia of Antrodia cinnamomea (EMAC). In recent years much attention has been paid to the alleged hepatoprotective activity of Antrodia cinnamomea which seems to be related to its antioxidant properties (Ao et al., 2009; Geethangili and Tzeng, 2009). Lu et al. (2007) reported that ethanolic extract of the mycelia of Antrodia cinnamomea showed hepatoprotective activity against ethanol-induced liver injury in rats. However, the molecular mechanism underlying such hepatoprotective efficacy was poorly understood. To elucidate the molecular mechanism, acute ethanol intoxicated liver injury and the hepatoprotective effects of EMAC were evaluated using male ICR mice. Increased levels of serum ALT, AST and hepatocellular GSH depletion are the conventional indicators of liver injury (Lu et al., 2007). Our in vivo experiments also showed that EMAC significantly inhibits ethanol–induced serum ALT, AST and hepatocellular lipid peroxidation. Notably, EMAC enhanced antioxidant status by preventing GSH depletion. Histopathological observations further revealed that EMAC could reduce the incidence of liver lesions. We further demonstrated that EMAC pretreatment potentially protects cells from oxidative stress by regulating Nrf-2 activity and downstream antioxidant enzyme protein HO-1 expression in...
mouse liver tissues. Our data also demonstrated the involvement of MAPKs in activation of Nrf-2, a result which correlates with related reports (Surh, 2003; Dai et al., 2003). Histopathology of liver section showed ethanol treatment produced swelling and hydropic degeneration of hepatocytes. The EMAC treated groups showed minimal hepatic changes (Fig. 10). In contrast, hepatic swelling was observed in normal control mice. We assume that this swelling was due to overnight starvation, which is agreement with previous study (LeBouton, 1982).

In conclusion, this study demonstrates that pretreatment with antroquinonol leads to protection of hepatic cells against ethanol-induced oxidative stress through a mechanism that involves Nrf-2 activation and up-regulation of expression of its downstream antioxidant genes mediated by MAP kinase proteins. Very similar results were obtained in vivo with EMAC and an acute ethanol-induced mouse model. These results provide a scientific basis for the hepatoprotective effects of *Antrodia cinnamomea* and derived pure compound antroquinonol and suggest it may be of therapeutic value in alcoholic liver diseases.

Fig. 10. Effects of EMAC on mouse liver histopathology. Mice were pretreated with EMAC (250, 500 and 1000 mg/kg) or silymarin (200 mg/kg) once a day for 10 days. After 10 days, mice were administered 3 doses of 5 g/kg body weight of ethanol at 12 h intervals. Control mice received 2% of DMSO in dH$_2$O or ethanol alone. Mice were sacrificed 12 h after the final ethanol administration. Livers were harvested, formalin fixed, and stained with hematoxylin-eosin for evaluation of pathological changes. Ethanol-induced swelling and hydropic degeneration of hepatocytes (arrows) was observed. (A) Control; (B) ethanol alone; (C) EMAC-250 + ethanol; (D) EMAC-500 + ethanol; (E) EMAC-1000 + ethanol; (F) silymarin-200 + ethanol. Original magnification 400×.

Appendix A. Supplementary data


References


