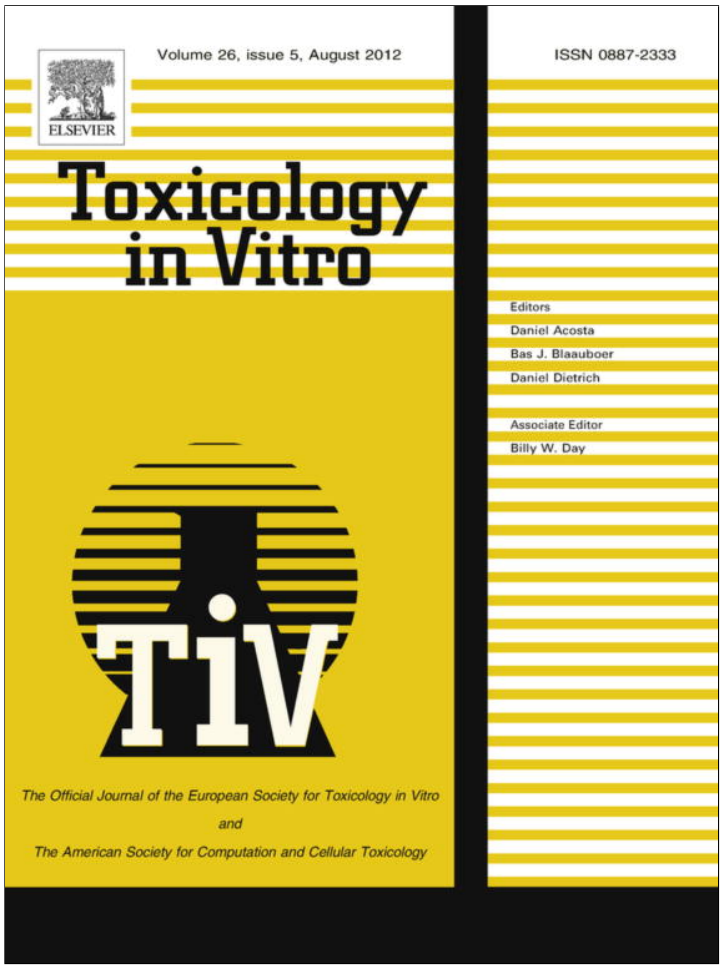

Toxicology in Vitro 26 (2012) 700–708

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Hepatoprotective effect of lucidone against alcohol-induced oxidative stress in human hepatic HepG2 cells through the up-regulation of HO-1/Nrf-2 antioxidant genes

K.J. Senthil Kumar [a], Jiunn-Wang Liao [b], Jun-Hong Xiao [a], M. Gokila Vani [a], Sheng-Yang Wang [a,c,*]

[a] Department of Forestry, National Chung Hsing University, Kou Kung Road, Taichung 402, Taiwan
[b] Graduate Institute of Veterinary Pathology, National Chung Hsing University, Kuo-Kung Road, Taichung 402, Taiwan
[c] Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan

ARTICLE INFO

Article history:
Received 14 November 2011
Accepted 22 March 2012
Available online 30 March 2012

Keywords:
Lucidone
Hepatoprotective
Oxidative stress
Heme oxygenase-1
NF-E2 related factor-2

ABSTRACT

Lucidone was previously reported to exhibit anti-inflammatory activity *in vitro* and *in vivo*. In the present study, we characterized the mechanisms underlying the hepatoprotective effect of lucidone against alcohol-induced oxidative stress *in vitro*. Human hepatoma (HepG2) cells were pretreated with lucidone (1–10 μg/mL) and then hepatotoxicity was stimulated by the addition ethanol (100 mM). With response to ethanol-challenge, increased amount of alanine aminotransferase (ALT) and aspirate aminotransferase (AST) release were observed, whereas lucidone pretreatment significantly inhibited the leakage of AST and ALT in HepG2 cells without appreciable cytotoxic effects. We also found that lucidone pretreatment significantly decreased ethanol-induced nitric oxide (NO), tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), reactive oxygen species (ROS) and glutathione (GSH) depletion in HepG2 cells. Furthermore, Western blot and quantitative-PCR analyses showed that ethanol-exposure apparently down-regulated endogenous anti-oxidant hemoxygenase-1 (HO-1) expression, whereas pretreatment with lucidone significantly up-regulates HO-1 expression followed by the transcriptional activation of NF-E2 related factor-2 (Nrf-2). Interestingly, the profound up-regulation of HO-1 and Nrf-2 were observed in only ethanol-challenged cells, which evidenced that lucidone-induced induction of HO-/Nrf-2 were specific with oxidative stress. Thus, we concluded that lucidone-mediated up-regulation of phase-II enzymes and HO-1 *via* Nrf-2 signaling pathway may provide a pivotal mechanism for its hepatoprotective action.

1. Introduction

In many part of the world, drinking alcoholic beverages is a common feature of social gathering. Nevertheless, over consumption of alcohol carries a risk of adverse health effects related to its intoxicating, toxic and dependence-producing properties (Anonymous, 2009). Despite the fact that acute and chronic liver diseases represent a global concern, modern medical treatments are often difficult to handle and have limited efficiency (Lee et al., 2007). Even though, past decades much attention has been made to understand the pathogenesis of alcoholic liver diseases, there is remain no effective therapy for this disease (Song et al., 2006). Recent studies has demonstrated that ethanol-induced oxidative stress and inflammation that produce vast amounts of cytokines and chemokines, especially nitric oxide, tumor necrosis factor-alpha, transforming growth factor-beta (TGF-β) and reactive oxygen species, which are believed to play a major role in pathogenesis and progression of alcoholic liver diseases (Song et al., 2006; Majano et al., 2004; Guitierrez-Ruiz et al., 1999; Nah et al., 2005).

Nrf-2 is a master regulator of ARE-driven antioxidant gene expression. Under the normal or unstressed condition, Nrf-2 is tethered in cytoplasm by another protein called Keap-1(Kelch-like ECH associated protein-1). Upon cell stimulation, Nrf-2 dissociate from Keap-1 and the unbound Nrf-2 translocate into the nucleus and where it bind to the ARE in upstream promoter region of many anti-oxidant genes, including HO-1, NQO-1, GSTA-2, γ-GCLC and γ-GCLM (Surh, 2003). Apart from the other anti-oxidant genes, HO-1 the inducible form of heme oxygenase plays a central role in iron homeostasis and anti-oxidant defense in living cells (Maines and Gibbs, 2005). There is increasing evidence that induction of HO-1 expression in liver has been considered to be a potential therapeutic target for various liver diseases (Farombi et al., 2008). In other hand, oxidative stress-induced by ethanol or acetaldehyde generates a marked elevation of lipid peroxidation and dramatically decreased GSH levels in human hepatic cell lines (Guitierrez-Ruiz

* Corresponding author at: Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan. Tel.: +886 4 22840345x138; fax: +886 4 22873628.
E-mail address: taiwanfir@dragon.nchu.edu.tw (S.-Y. Wang).

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.03.012

et al., 1999). Therefore, the restoration of GSH has been shown to inhibit the development of alcoholic liver damage and pathogenesis (Das and Vasudevan, 2007).

Considerable attention has been devoted in recent years to the development of complementary and alternative medicines for the treatment of liver diseases. Therapeutically effective agents developed from natural sources are particularly attractive for treatments as they have the potential to reduce the risk of drug toxicity (Batey et al., 2005). Several natural antioxidants are able to exert protective effects not only free radical scavenging, but also augment expression of cytoprotective and/or antioxidant genes *via* Nrf-2 driven ARE signaling pathway (Surh, 2003).

Lucidone is a naturally occurring cyclopentenedione, which was initially isolated from the fruits of *Lindera lucida* (Lauraceae) and subsequently from other species such as *Lindera erythrocarpa* (Kumar et al., 2010). It has been reported that lucidone inhibited human farnesyl protein transferase activity with an IC_{50} value of 40 ± 3.5 μM (Oh et al., 2005). The anti-inflammatory activity of *L. erythrocarpa* fruits has been preliminarily evaluated by our team. Four anti-inflammatory cyclopentenediones were identified by bioactivity-guided fractionation, between which lucidone was the strongest inhibitor of NO production. Meanwhile, lucidone was also a potent anti-inflammatory agent in a croton oil-induced mouse ear edema assay (Wang et al., 2008). Also, our recent studies suggest that lucidone inhibits LPS-induced inflammation *in vitro* and *in vivo* models (Kumar and Wang, 2009; Kumar et al., 2010). To our knowledge, no studies have been investigated the alcohol-induced hepatotoxicity and hepatoprotective efficacy of lucidone. Thereby, the present study was carried out in order to understand the molecular mechanism underlying the anti-oxidant and hepatoprotective activity of lucidone in an *in vitro* cell culture model.

2. Materials and methods

2.1. Chemicals and reagents

Lucidone was isolated from the fruits of *Lindera erythrocarpa* Makino as described previously (Wang et al., 2008), and the purity was >99% verified by HPLC and ^{1}H NMR. Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA). Gries reagents, curcumin, 2′-7′-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA), and mouse monoclonal anti-β-actin antibody was obtained from Sigma–Aldrich (St. Louis, CA). Mouse monoclonal HO-1, rabbit polyclonal Nrf-2, and rabbit polyclonal Histone-H3 antibodies were purchased from Abcam (Cambridge, MA). All other chemicals and solvents used in this study were reagent or HPLC grade.

2.2. Cell culture and sample treatment

Human hepatoma (HepG2) cell line was purchased from the American type culture collection (ATCC, Manassas, VA) were cultured in MEM medium supplemented with 10% FBS, 1% glucose, 1% glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 in air at 37 °C. HepG2 cells were incubated in the presence or absence of different concentrations of lucidone (1–20 μg/mL) or curcumin (10 μg/mL) and then the hepatotoxicity was stimulated by the addition of ethanol (10–500 mM) for 24 h. Commercially available curcumin was used as a reference hepatoprotective agent (Bao et al., 2010) for this study.

2.3. Cell viability assay

Cell viability was determined using colorimetric MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay as described previously (Fotakis and Timbrell, 2006). In brief, HepG2 cells (1×10^5 cells/mL) were cultured in 96 well plate with or without various concentrations of lucidone (1–20 μg/mL) and ethanol (10–500 μM) for 24 h. After 24 h incubation, medium was removed and 10 μL of MTT (10 μg/mL) in 90 μL of fresh medium was added to each well and incubated at 37 °C for further 2 h. Subsequently 100 μL of dimethylsulfoxide (DMSO) was added to dissolve formazan crystals and the absorbance was measured at 570 nm using ELISA microplate readed (μQuant, Bio-Tek instruments Inc., Winooski, VT) and the percentage of cell viability was calculated.

2.4. Determination of hepatic ALT and AST production

Hepatic ALT and AST levels in culture medium were measured by commercially available assay kits (Randox Laboratories, Antrim, UK). Briefly, HepG2 cells (1×10^6 cells/dish) in 6 cm dish were incubated in the presence or absence of various concentrations lucidone (1, 5 and 10 μg/mL) or curcumin (10 μg/mL) for 1 h then the cells were stimulated with ethanol (100 mM) for 24 h. Control cells received 0.1% of DMSO instead of lucidone or curcumin. After incubation, culture supernatant was removed and the total ALT and AST levels were measured using an ELISA microplate reader at 340 nm according to the supplier instructions.

2.5. Determination of lipid peroxidation and reduced glutathione

Accumulated intracellular lipid peroxidation was measured as MDA equivalent generated, as an indicator of lipid peroxidation in cultured cell lysates (Esterbauer et al., 1991). Lipid peroxidation assay kit (Oxford Biomedical Research, Oxford, MI) was used to quantify ethanol-induced cellular lipid peroxidation followed by the supplier's instructions. In brief, HepG2 cells (1×10^6 cells/dish) were cultured in 6 cm dish and incubated with or without various concentrations of lucidone (1, 5 and 10 μg/mL) or curcumin (10 μg/mL) for 1 h then the cells were stimulated by the addition of ethanol (100 mM) for 24 h. Control cells received 0.1% of DMSO instead of lucidone or curcumin. In parallel assay, cellular GSH levels also measured in non-protein hepatoma lysates using commercially available GSH assay kit (Oxis International, Foster City, CA).

2.6. Determination of NO and TNF-α production

The concentration of NO in culture supernatant was measured in the form of nitrite, a major stable product of NO using Griess reagents assay as described previously (Wang et al., 2008). Briefly, HepG2 cells (1×10^5 cells/mL) were seeded in 96 well plate and incubated in the presence or absence of various concentrations of lucidone (1, 5 and 10 μg/mL) or curcumin (10 μg/mL) for 1 h. Hepatotoxicity was stimulated by the addition of ethanol (100 mM) for 24 h. After incubation, culture supernatant was collected, 100 μL of culture supernatant was mixed with equal amount of Griess reagents and the NO production was measured by ELISA microplate reader. In parallel assay, the level of hepatic TNF-α secretion in culture medium was measured using commercially available human TNF-α assay kit (Biosource, Camarillo, CA). Total culture supernatant was diluted with working reagent (1:2); 100 μL of each diluted sample was used for ELISA assay (Lin et al., 2009). The total secreted amount of TNF-α levels were estimated by absorbance at 450 nm by using ELISA micro-plate reader.

2.7. Measurement of intracellular ROS accumulation

Total intracellular ROS accumulation was monitored by fluorescence marker DCF-DA as described previously (Nah et al., 2005) with minor modifications. HepG2 cells (5×10^4 cells/well) were seeded in 24 well plate and pre-incubated with various concentrations of lucidone (1, 5 and 10 μg/mL) or curcumin (10 μg/mL) for 1 h, then the cells were stimulated with ethanol (100 mM) for 1 h. After incubation, culture supernatant was removed and cells were rinsed with phosphate buffer saline (PBS). $DCFH_2$-DA (10 μM) in Kreb's buffer was added into the culture plate and incubated for 30 min. Stained cells were examined under fluorescence spectrophotometer (Hidex Oy, Turku, Finland) at 488 nm/530 nm. Intra cellular ROS generation was measured by the DCF fluorescence. The percentage of DCF fluorescence was compared with untreated control, which was arbitrarily assigned 100%. The assay was performed in triplicate.

2.8. Protein extraction and western blot analysis

HepG2 cells (1×10^6 cells/dish) were seeded in 6 cm dish and pretreated with various concentrations of lucidone (1, 5 and 10 μg/mL) or curcumin (10 μg/mL) for 1 h and the hepatotoxicity was induced by the addition of ethanol (100 mM) for 2–24 h. Control cells received 0.1% DMSO instead of drug or ethanol. Cytoplasmic and nuclear fractions were extracted by commercially available nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL). Protein concentration was determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Bovine serum albumin (BSA) was used as a standard protein. Western blot analysis was performed with appropriate antibodies with ECL Western blot reagents (Millipore, Billerica, MA) and the image was visualized by VL Chemi-Smart 3000 (Viogene Biotek, Sunnyvale, CA) image documentation device. Densitometry analyses were performed by commercially available quantitative software (Quantitative One, Bio-Rad Laboratories, Hercules, CA).

2.9. Electrophoretic mobility shift assay (EMSA)

EMSA was performed to determine Nrf-2 ARE binding activity using commercially available fluorescence-based EMSA kit (Invitrogen, Carlsbad, CA). In brief, oligonucleotide probes: human anti-oxidant responsible element (hARE), forward primer 5′-CTC GAG CCC TAT AAC TGC TAT CTC-3′, reverse primer 5′-AAG CTT GGC TCT GGT GCA GTC CGG-3′ (Lee et al., 2009) were synthesized by TRI Biotech (Taipei, Taiwan), and then annealed with TE buffer for 5 min at 94 °C then gradually cooled for 3 h. Nuclear extract (20 μg) was incubated with 20 ng of double-standard hARE probe for 30 min at room temperature along with binding buffer. DNA protein complex was separated by 6% native polyacrylamide gel electrophoresis (PAGE) and the complex was visualized using fluorescence-based EMSA reagent, and the luminescence intensity was quantitated by VL Chemi-Smart 3000 imaging device.

2.10. RNA extraction and quantitative real-time PCR analysis

Total RNA extraction and RT-PCR analysis was performed as described previously (Kumar and Wang, 2009). In brief, HepG2 cells (1×10^6 cells/dish) were seeded in 6 cm dish and pretreated with various concentrations of lucidone (1, 5 and 10 μg/mL) or curcumin (10 μg/mL) for 1 h and hepatotoxicity was induced by the addition of ethanol (100 mM) for 8 h. Total RNA was extracted by TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Quantitative-PCR analysis was performed using Applied Biosystems detection instruments and software. Forward and reverse primers (10 mM) were incorporated with SYBR green working solution (Invitrogen, Carlsbad, CA), used as a PCR master mix, 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 used as an internal standard 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 CGA CAG CAT GC-3′; β-actin, forward 5′-ACC CAC ACT GTG CCC ATC TA-3′, reverse 5′-CGG AAC CGC TCA TTG CC-3′ (Liu et al., 2004a,b). The copy number of each transcript was calculated as the relative copy number, normalized by β-actin copy number.

2.11. Statistical analysis

The results were expressed as means ± standard deviations of the three independent experiments. Statistical analyses of the results were made using Dunnett's test for multiple comparisons and Student's *t*-test for single comparison. Significant differences ($^{*}P < 0.05$ and $^{**}P < 0.01$ and $^{***}P < 0.001$) considered significant from ethanol *vs* sample. $^{\#}P < 0.01$ control *vs* ethanol.

3. Results

3.1. Cytotoxic effects of lucidone on human hepatoma HepG2 cells

Prior to *in vitro* hepatoprotective studies, the cytotoxic effect of lucidone was examined in human hepatoma HepG2 cells. Cells (1×10^5 cells/mL) were incubated with various concentrations of

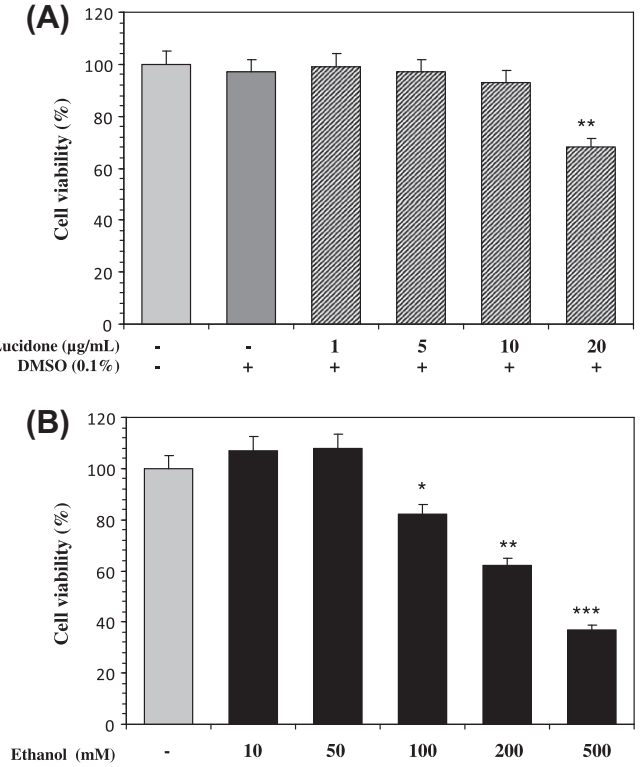

Fig. 1. Cytotoxic effect of lucidone and ethanol on cultured human hepatoma HepG2 cells. Cell viability was measured by MTT assay as described in Section 2. Cells were treated with indicated concentrations of lucidone and ethanol for 24 h. (A) Cells treated with various concentrations of lucidone for 24 h. (B) Cells were treated with indicated concentrations of ethanol for 24 h. Results are expressed as percent control and represent means ± standard deviations (SD) of three independent experiments. Percentage of viable cells was calculated with control cells. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ was considered significant for control cells.

lucidone (1, 5, 10 and 20 μg/mL) for 24 h. Results of MTT assay showed that <97 ± 3% of cells were survived within the treatment concentrations of 10 μg/mL of lucidone, whereas over the concentration of 10 μg/mL cell survival was eventually fall-down into >70 ± 2% (Fig. 1A). In parallel MTT assay, incubation of HepG2 cells with ethanol resulted in a dose-dependent decrease of cell viability. Survival of hepatic cells after 24 h exposure to ethanol at 10, 50, 100, 200 and 500 mM were 107%, 108%, 82%, 62% and 37%, respectively (Fig. 1B). Moreover, cells exposed to >100 mM of ethanol, survived 100 ± 8.5% for 24 h and the significant ($P < 0.05$) reduction of cell survival manifested with <100 mM of ethanol (Fig. 1B). Therefore, the non-cytotoxic concentrations of lucidone (1–10 μg/mL) and cytotoxic concentration of ethanol (100 mM) were selected as the standard concentrations for the subsequent assessments.

3.2. Lucidone pretreatment inhibits ethanol-induced hepatic enzyme release and lipid peroxidation in HepG2 cells

In vitro hepatotoxicity can be directly determined by measuring levels of hepatic transaminase release into the culture medium (Dambach et al., 2005). Incubation of hepatic cells with ethanol for 24 h, an increase in intercellular ALT and AST leakage was observed compared to control cells, whereas lucidone pretreatment significantly attenuated ethanol-induced elevation of ALT and AST levels in a dose-dependent manner (Fig. 2A and B). Furthermore, we examined the effects of lucidone on ethanol-induced lipid peroxidation, as measured by MDA level in culture lysates. Ethanol-exposure for 24 h significantly ($P < 0.001$) increased intracellular MDA level (47 ± 4.3 μM) compared to control (6 ± 2.6 μM) in HepG2 cells (Fig. 2C). However, the elevated MDA levels (47 ± 4.1 μM) were significantly descended to 13 ± 2 and 9 ± 1.3, and 7 ± 1.2 at 1, 5, and 10 μg/mL of lucidone, respectively (Fig. 2C). On the other hand, the effects of lucidone on ethanol-induced hepatic GSH depletion were monitored. Because, during the oxidative stress endogenous GSH was consumed and resulted in rapid depletion of GSH in hepatic cells (Eklow-Lastbom et al., 1986). In agreement with this notion, Fig. 2D showed that ethanol-exposure declined intracellular GSH level (83 ± 6.3 nM) compared with the control cells (121 ± 4.1 nM), whereas pretreatment with lucidone significantly prevents ethanol-mediated hepatic GSH depletion, as evidenced by the restoration of GSH to basal level (120 ± 3.3 mM and 129 ± 2.7 nM at the concentrations of 5 and 10 μg/mL, respectively). In addition, we observed that ethanol-induced hepatic ALT, AST, MDA and GSH depletion were significantly inhibited by curcumin, which was used as a positive drug control in this study (Fig. 2).

3.3. Lucidone pretreatment inhibits ethanol-induced NO and TNF-α production in HepG2 cells

Ethanol and its metabolites directly induce inflammatory response mediated by the production of pro-inflammatory cytokines and chemokines (Guitierrez-Ruiz et al., 1999). In the present study, we also have observed that ethanol exposure certainly produced significant amount of NO in cultured HepG2 cells. Whereas, pretreatment of cells with lucidone (1–10 μg/mL) resulted in significant ($P < 0.01$–0.001) and dose-dependent inhibition of NO

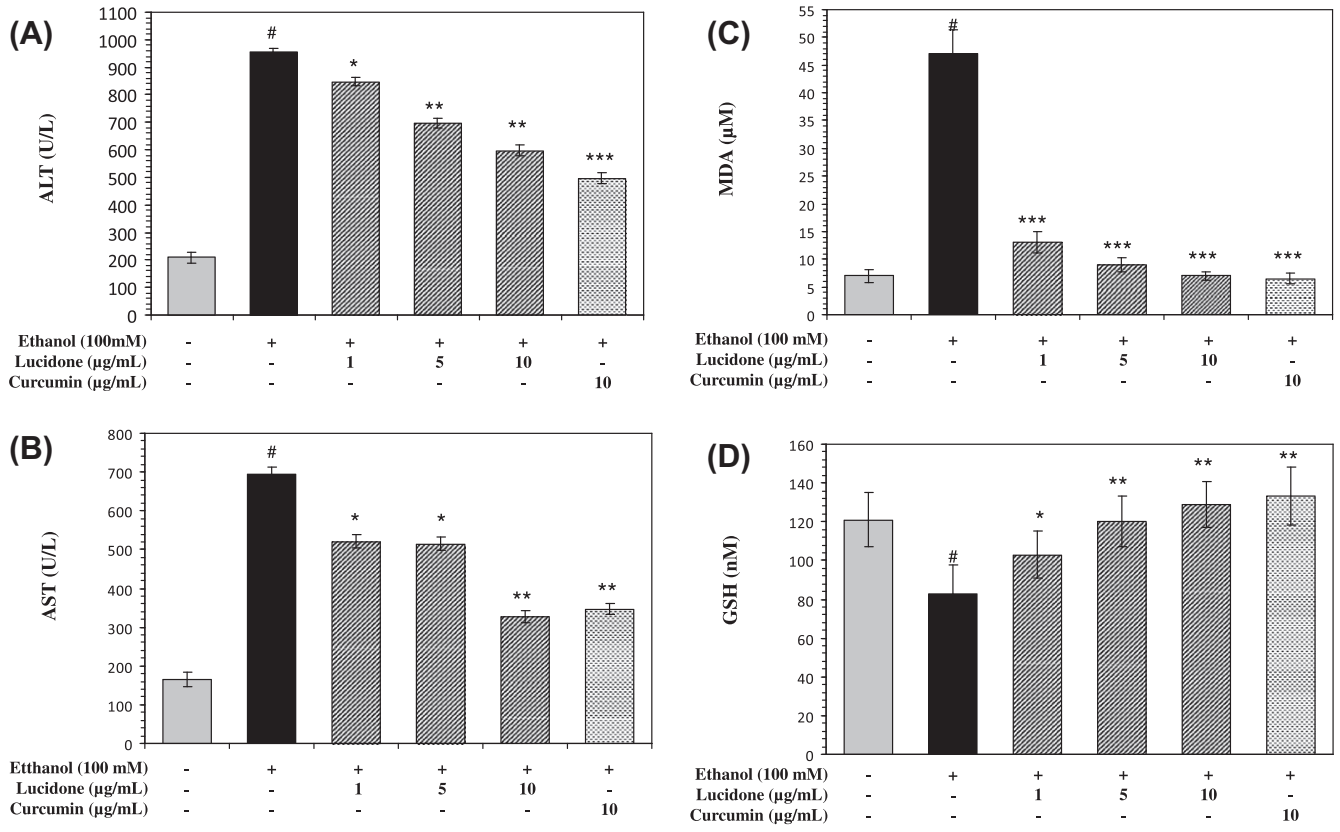

Fig. 2. Lucidone down-regulates ethanol-induced ALT, AST, MDA and GSH depletion in HepG2 cells. Cells were pretreated with indicated concentrations of lucidone and curcumin for 1 h and then oxidative stress was stimulated by addition of ethanol for 24 h. (A) ALT, (B) AST released by hepatic cells was measured in the culture supernatant. (C) Hepatocellular MDA and (D) GSH depletion was assessed from HepG2 cell lysates. The histogram shown in this figure is from one representative experiment that was performed in triplicate with similar results. Each value is expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant for EtOH alone. $^{\#}P < 0.05$ was considered significant for control *vs* EtOH alone.

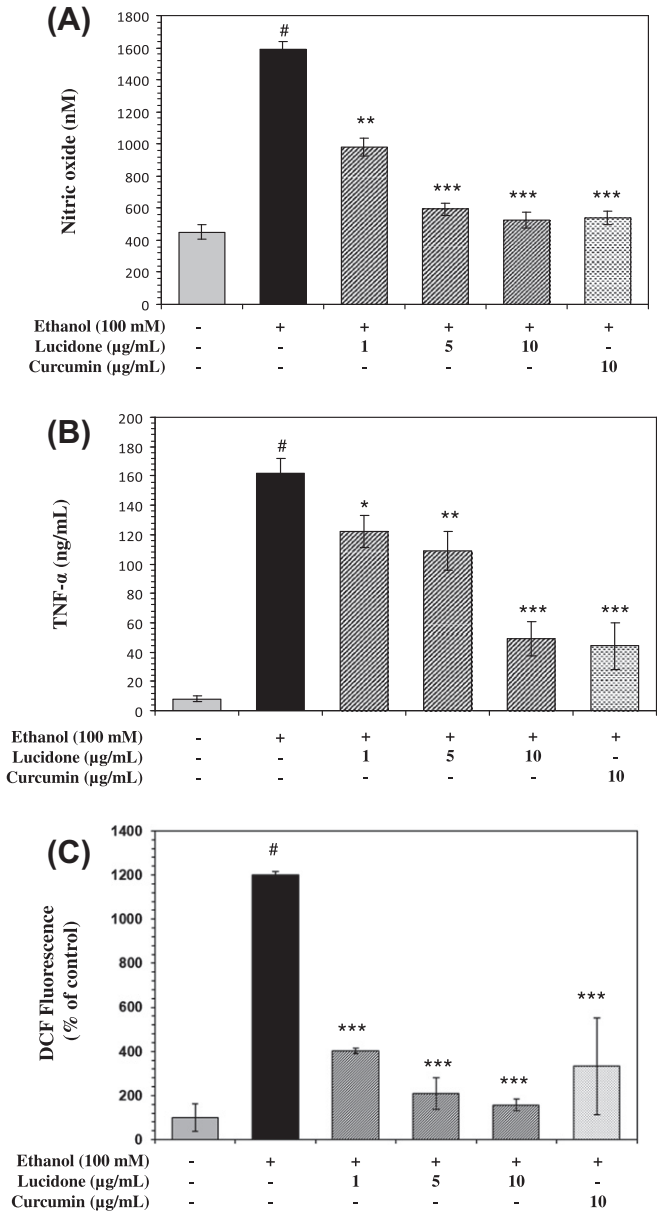

Fig. 3. Lucidone suppressed ethanol-induced NO, TNF-α and ROS production in HepG2 cells. (A) NO production was monitored in culture supernatant by Griess assay. (B) Hepatic cell released TNF-α level in cultured media was quantified by commercially available EIA kit. (C) Hepatocellular ROS accumulation was monitored by using $DCFH_2$-DA florescence method as described in materials and methods. The histogram shown in this figure is from one representative experiment that was performed in triplicate with similar results. Each value is expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant for EtOH alone. $^{\#}P < 0.05$ was considered significant for control *vs* EtOH alone.

production were observed (Fig. 3A). To our knowledge, this is the first report indicating that ethanol-exposure could induce NO production in cultured HepG2 cells. In addition, we found that ethanol-exposure significantly ($P < 0.001$) provoked TNF-α secretion (163 ± 10.7 ng/mL) compared with the control cells (8 ± 1 ng/mL). Pretreatment with lucidone (1, 5 and 10 μg/mL) significantly ($P < 0.05$–0.001) attenuated ethanol-induced TNF-α secretion in a dose-dependent manner 122 ± 1, 110 ± 3, 46 ± 2 ng/mL, respectively (Fig. 3B). The inhibitory effect of lucidone on NO and TNF-α production were comparable with that of the standard drug curcumin.

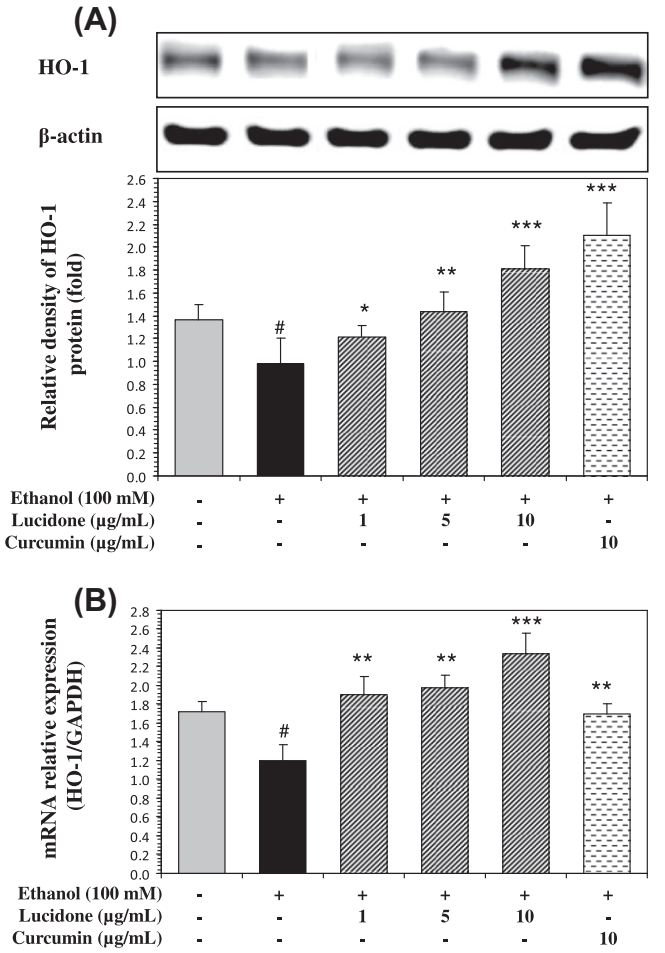

Fig. 4. Lucidone up-regulates HO-1 expression in ethanol-induced HepG2 cells. (A) Cytosolic lysates were prepared and subjected to Western blot analysis to determine the HO-1 protein expression level in ethanol-induced HepG2 cells. β-Actin was used as an internal control. HO-1 protein was normalized by β-Actin. (B) RT-PCR analysis was performed to monitor HO-1 mRNA expression level using commercially available SYBR green dye. HO-1 mRNA was normalized by GAPDH. The photomicrograph and densitometry graph shown in this figure are from one representative experiment that was performed in triplicate with similar results. Each value is expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant for EtOH alone. $^{\#}P < 0.05$ was considered significant for control *vs* EtOH alone.

3.4. Lucidone pretreatment prevents ethanol-induced ROS generation in HepG2 cells

Sustained intracellular ROS production has been recognized as a crucial step for ethanol-induced oxidative stress in hepatic cells. Further, to investigate the effects of lucidone on ethanol-induced ROS generation in hepatic cells, we utilized the well-characterized $DCFH_2$-DA fluorescence assay. Compared to un-stimulated control cells (100 ± 63%), ethanol exposure markedly increased $DCFH_2$ fluorescence (1200 ± 15%) over a 1 h course of time. Cells that were pre-treated with lucidone significantly ($P < 0.001$) prevents ethanol-induced intracellular ROS generation in HepG2 cells (Fig. 3C). Curcumin is known for its anti-oxidant effect and was used as a positive drug control that also exhibits significant ROS inhibition (333 ± 219%) at the concentration of 10 μg/mL.

3.5. Lucidone augmented HO-1 mRNA and protein expression in ethanol-induced HepG2 cells

It has been well documented that HO-1, a major antioxidant enzyme that plays an important role against ethanol-induced oxida-

tive stress in hepatic cells (Bao et al., 2010). Therefore, we hypothesized that the inhibitory effect of lucidone against ethanol-induced oxidative stress could be mediated by the induction of antioxidant genes including HO-1 and NQO1. Results of Western blot and RT-PCR analysis confirmed that pretreatment of cells with lucidone significantly ($P < 0.05–0.001$) augmented HO-1 protein and mRNA expression in a dose-dependent manner (Fig. 4A and B). Moreover, a statistically significant ($P < 0.05$) reduction of HO-1 expression was observed when cells were incubated with ethanol (Fig. 4A). Curcumin also enhanced HO-1 expression in protein level, whereas the level of mRNA was not affected (Fig. 4A and B). In contrast, cells pre-incubated with neither lucidone nor curcumin were found no significant effect on NQO1 mRNA or protein expression in ethanol-challenged HepG2 cells (data not shown).

3.6. Lucidone pretreatment enhanced Nrf-2 activity in ethanol-induced HepG2 cells

HO-1 can be activated by Nrf-2, a major transcription factor that regulates ARE-driven phase-II gene expression (Surh, 2003). Therefore we attempted to determine whether lucidone could activate Nrf-2 in association with its HO-1 up-regulation in HepG2 cells. Result of Western blot analysis showed that lucidone treatment significantly ($P < 0.05–0.001$) induced Nrf-2 nuclear translocation, as

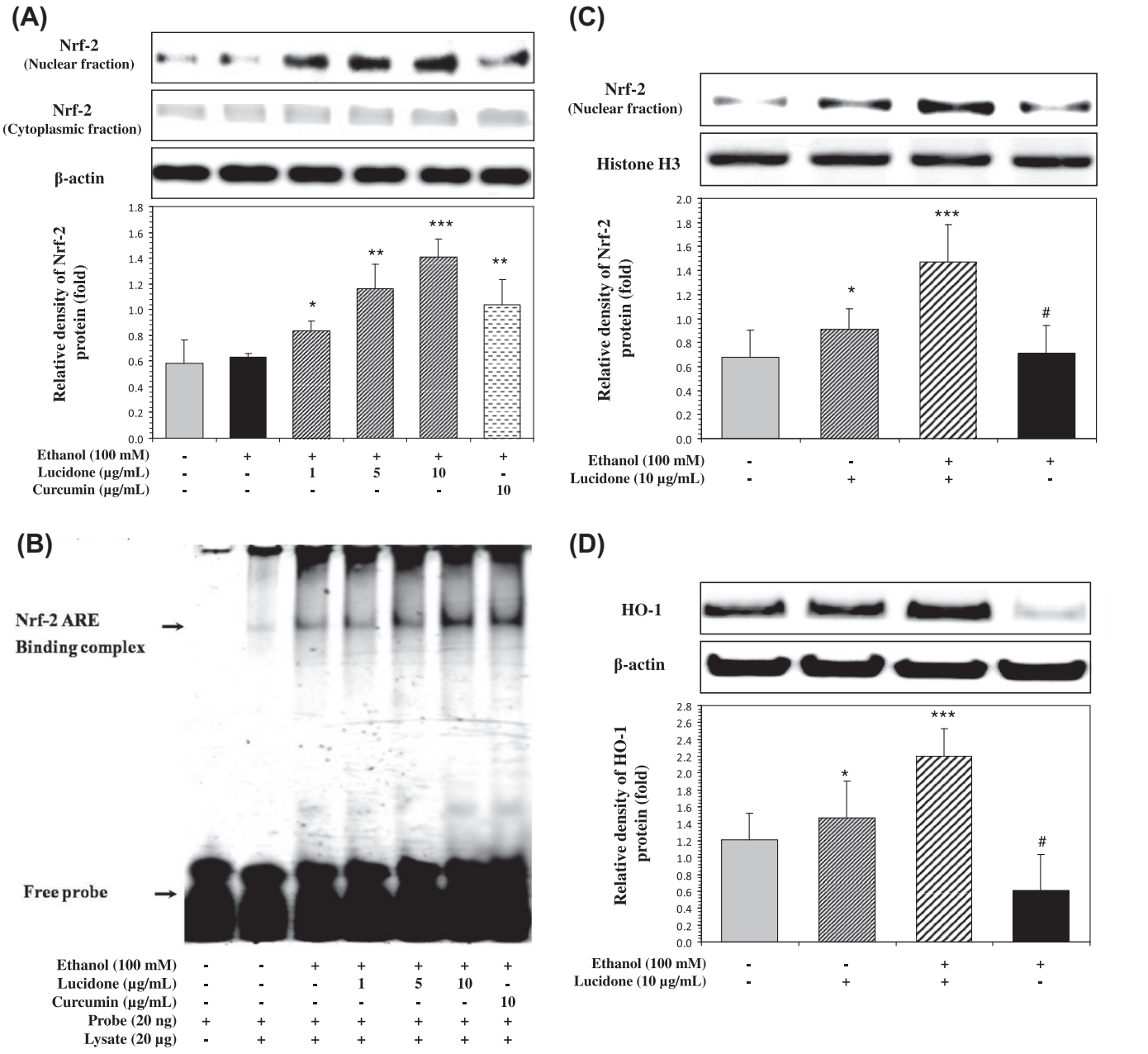

Fig. 5. Lucidone enhanced Nrf-2 nuclear translocation and ARE binding activity in ethanol-induced HepG2 cells. (A) Nuclear lysates were prepared and subjected to Western blot analysis to quantify the lucidone-induced nuclear translocation of Nrf-2. β-Actin was used as an internal control. (B) EMSA was performed to monitor Nrf-2 ARE binding activity in nuclear fraction. (C and D) Nuclear (Nrf-2) and cytoplasmic (HO-1) lysates were subjected to Western blot analysis to quantify the lucidone-induced up-regulation of Nrf-2 and HO-1 proteins in ethanol-induced HepG2 cells. Histone H3 for nuclear fraction and β-Actin for cytoplasmic fraction was used as an internal control. The photomicrograph and densitometry graph shown in this figure are from one representative experiment that was performed in triplicate with similar results. Each value is expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant for EtOH alone. $^{\#}P < 0.05$ was considered significant for control *vs* EtOH alone and $^{\varphi}P < 0.001$ was considered significant from sample *vs* ethanol group.

evidenced by the increased amount of Nrf-2 were found in nucleus, whereas the amount of cytosolic Nrf-2 was unaffected by neither lucidone nor curcumin (Fig. 5A). Further to elucidate the role of Nrf-2 in transcriptional activation, Nrf-2 ARE binding activity was monitored by using EMSA with oligonucleotides harboring HO-1 specific ARE sequence. As shown in Fig. 5B, lucidone pretreatment significantly increased Nrf-2 ARE binding activity in a dose-dependent manner. Interestingly, when compared with un-treated control group, lucidone pretreatment significantly ($P < 0.001$) increased nuclear translocation of Nrf-2 followed by the induction of HO-1 in HepG2 cells; although the profound augmentation of HO-1 and Nrf-2 was observed when cells challenged with ethanol (Fig. 5C and D). Indeed, this data confirmed that lucidone-induced up-regulation of HO-1 and Nrf-2 expression is critically governed by the ethanol-induced oxidative stress in hepatic cells.

4. Discussion

Since oxidative stress is a putative mechanism underlying alcoholic liver diseases, using antioxidants to ameliorate oxidative stress and alleviate ethanol hepatotoxicity is a logical approach. This approach has shown high rate of success in both *in vitro* and *in vivo* models. Previously, we have demonstrated that antioxidants antroquinonol and sylimerin protect cultured hepatic cells against ethanol-induced oxidative stress by down-regulating hepatic transaminase enzymes and intracellular ROS generation (Kumar et al., 2011). Similarly, pretreatment of hepatocytes with quercetin provided protection against ethanol-induced oxidative stress by downregulating hepatic transaminase enzymes (Liu et al., 2010). Green tea polyphenols quercetin, EGCG, catechin and betaine is demonstrated to prevent ethanol-induced CYP2E1 and GPX4 expression in human hepatoma HepG2 cells (Oliva et al., 2011). Peng et al. (2010) showed that β-carotene decreased oxidative stress and prevented ethanol-induced cell death by inhibiting apoptotic proteins caspase-9 and caspase-3 expression. In a model of chronic ethanol exposure, administration of Vitamin E and antioxidant resveratrol effectively ameliorate ethanol-induced oxidative challenges by down-regulating transaminase enzymes including AST, ALT, IL-10, TNF-α, IFN-γ, and TGF-β1 in the serum. (Das et al., 2010). Administration of antioxidants ascorbic acid, quercetin, and thiamine has been found to block chronic ethanol-induced transaminase enzymes in the mice liver and serum (Ambadath et al., 2010). In a binge-alcohol administrated model, treatment of antioxidant carnosine significantly reduced ethanol-induced oxidative stress in the liver (Artun et al., 2010). These results suggest that oxidative stress critically contribute to ethanol hepatotoxicity, antioxidant protection is the effective strategies to prevent alcohol liver diseases.

The notable signs of ethanol-intoxicated hepatic injury are leakage of hepatic transaminases and cytokines into the circulation (Baldi et al., 2003). In the present study, we employed human hepatoma (HepG2) cells *in vitro* and found that incubation of hepatic cells with ethanol led to sustained increase of ALT and AST leakage from the cytoplasm into culture medium, which is agreement with other's observations (Neuman et al., 1993; Kaur et al., 2009; Khanal et al., 2009). Our result showed that pretreatment with lucidone significantly inhibits ethanol-induced ALT and AST release in a dose-dependent manner. In addition, the well known anti-oxidant curcumin also inhibits ethanol-induced ALT and AST release in HepG2 cells. This data were support a previous study by Rajakrishanan et al. demonstrated that curcumin as a potent hepatoprotective agent for alcohol intoxication (Rajakrishanan et al., 1998).

Reduced glutathione content is often used to evaluate oxidative stress in biological systems and augmentations of GSH/GSSG ratio have been demonstrated to protect liver from oxidative stress (Das and Vasudevan, 2007). Results of this study showed that lucidone pretreatment significantly prevents ethanol-induced GSH depletion and lipid peroxidation in HepG2 cells. A similar set of the results were also found in curcumin pretreated cells. Although, alcohol consumption results an excessive generation of free radicals such as superoxide radicals, free radicals, hydroxyl radicals and hydrogen peroxides. Ethanol-mediated ROS generation play a central role in the development of alcoholic liver diseases and limiting the expression of cytoprotective genes (Das and Vasudevan, 2007; Motohashi et al., 2010). Therefore, the removal of ROS accumulation through cellular anti-oxidant defense system could be maintaining intracellular redox homeostasis. We found that lucidone pretreatment significantly prevented ethanol-induced intracellular ROS accumulation in cultured HepG2 cells. Moreover, ethanol-mediated free radical formation has a great potential to react with lipid molecules, which leads to lipid peroxidation and the uncontrolled lipid peroxidation is known to be one of the manifestations of oxidative cell injury enhanced by ethanol ingestion. During the alcohol metabolism, alcohol oxidized into aldehydes especially acetaldehydes and malonaldehydes (MDA). Elevated MDA, decreasing anti-oxidant capacity has been used as a biomarker of oxidative stress (Guitierrez-Ruiz et al., 1999). We also observed that ethanol exposure sustained MDA level in cultured HepG2 cells; whereas the increases of MDA level were significantly inhibited by lucidone pretreatment. We believe the hydroxyl group located in the 3rd position may contribute to its potent anti-oxidant effects. Thus, the inherent antioxidant potential of lucidone may help to prevent ethanol-induced ROS generation and/or lipid peroxidation.

The crucial role of pro-inflammatory molecules such as NO, TNF-α and IL-1β has been indicated in both clinical and experimental alcoholic liver diseases (Zhao et al., 2008). In particular, TNF-α is of great relevance to liver pathology, as its expression and levels increase in many forms of liver diseases, such as alcoholic liver diseases become it mediated hepatocyte apoptosis (Fernandez-Checa et al., 2005). Mice deficient with TNF-α receptor (TNFR1), failed to develop alcoholic liver diseases except steotosis (Fernandez-Checa et al., 2002). Thus, the inhibition of TNF-α is primarily implied to prevent alcoholic liver diseases in human subjects. It is noteworthy that lucidone pretreatment significantly prevented alcohol-induced TNF-α production in cultured human hepatic cells. This observation was concomitant with our previous report that lucidone pretreatment inhibits LPS-induced TNF-α production in macrophage cells (Kumar and Wang, 2009). In addition to the critical role of pro-inflammatory molecules, NO has been pointed as another key player of liver diseases (Kirkali et al., 2000). A previous report described that ethanol-exposure induced NO formation in human astrocytoma cells (Davis et al., 2002). Then latter, Majano et al. (2004) reported that mixture of proinflammatory cytokines induces NO production in human hepatocyte HepG2 cells. An *in vivo* animal studies also have shown that acute or chronic alcohol exposure increased NO production in rat circulating system (Deng and Deitrich, 2007). However, there was no study revealed the ethanol-induced NO production in HepG2 cells. We therefore monitored ethanol-induced NO production in HepG2 cells. As we expected, NO production in culture media were markedly rose with response to ethanol-exposure, whereas the reduced amount of NO production were observed in lucidone pretreated cells. With best of our knowledge, this is the first report indicating ethanol-induced NO production in HepG2 cells. This data were well correlated with our previous report that lucidone inhibited LPS-induced NO production in murine macrophage cells (Kumar and Wang, 2009).

HO-1 is a rate limiting enzyme that catalyzes heme into biliverdin, free iron and carbon monoxide. Increased HO-1 expression has been reported to reduce cellular injury such as oxidative stress, pro-inflammatory cytokines production and the activation of pro-

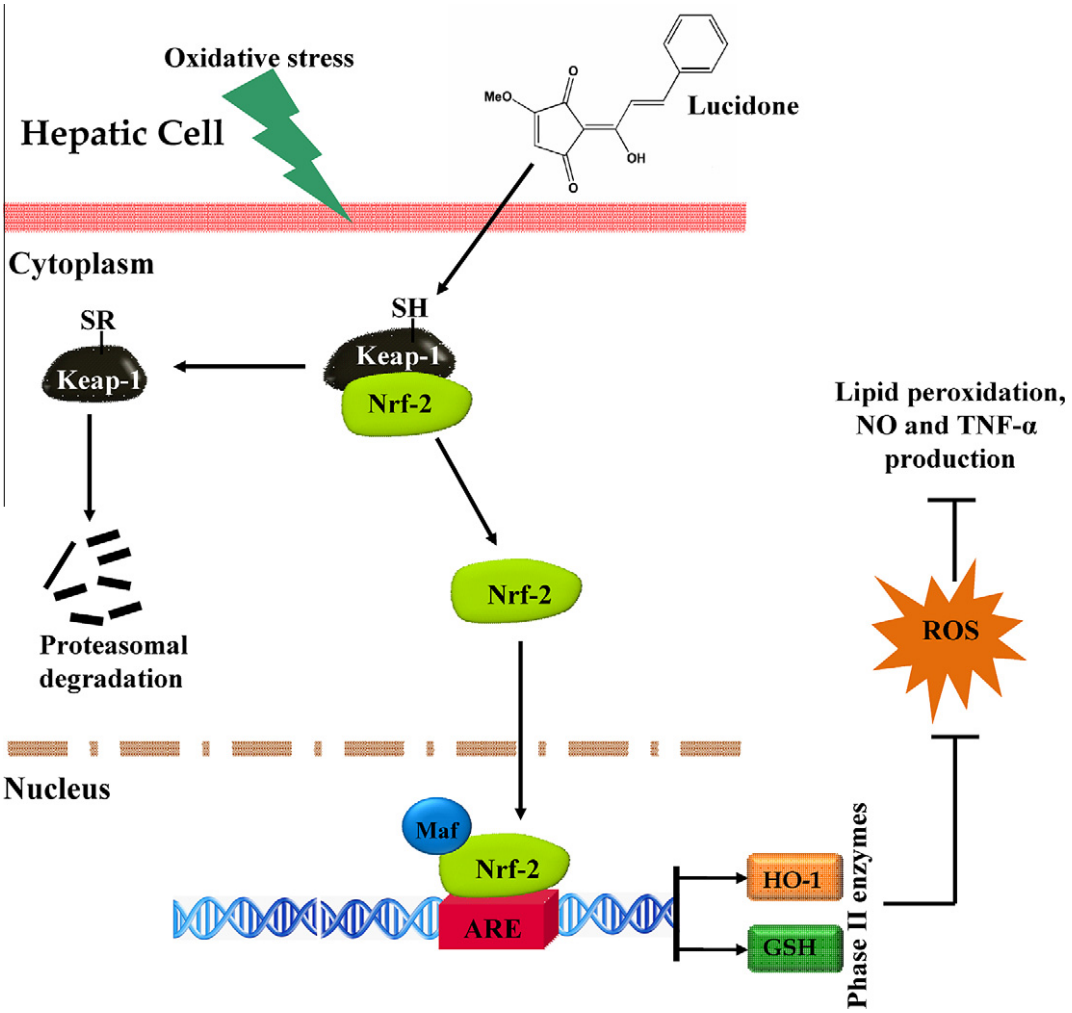

Fig. 6. Schematic representation depicting the mechanism by which lucidone-induced antioxidative response in human hepatic cells.

apoptotic inducers (Braudeau et al., 2003). A recent study reported that the ethanol-exposure markedly declined endogenous HO-1 level in hepatocytes (Bao et al., 2010). Our data also agreed that the ethanol treatment markedly reduced HO-1 protein and mRNA expression in cultured HepG2 cells. However, the ethanol-induced HO-1 decline was significantly prevented by lucidone pretreatment. Furthermore, Nrf-2, a transcription activator binds to ARE in the upstream promoter region of many anti-oxidant genes including HO-1 (Surh, 2003). Previous studies have shown that increasing Nrf-2 activity in hepatic tissues is highly hepatoprotective during chemical or ethanol-induced oxidative stress (Farombi et al., 2008; Yao et al., 2007). Our result showed that lucidone treatment significantly increased Nrf-2 activity by means of its nuclear translocation. Furthermore, results of EMSA also confirmed that pretreatment with lucidone not only up-regulates nuclear translocation, but also induce Nrf-2 transcriptional activity in ethanol-induced HepG2 cells. Surh (2003) reviewed that during oxidative condition, activation of Nrf-2 cascade was critically associated with their up-stream regulators including JNK, p38, ERK and PI3K. Therefore, we monitored the expression of MAP kinase proteins (JNK1/2, ERK1/2 and p38) in the same treatment conditions. However, there were no significant up-regulation of these MAPKs, rather than lucidone inhibits their expression in HepG2 cells (data not shown) which was closely resembled in our previous report (Kumar and Wang, 2009). Thus, we suggest that the activation of Nrf-2 by lucidone is may be MAPKs independent mechanism. In other hand, the structural activity relationship studies revealed that phytocompounds that possessed α, β-unsaturated ketone moiety can act as Michael-reaction acceptors that are able to modify cystine thiols located in Keap-1, which is a major Nrf-2 inhibitor (Surh, 2003). It has been extensively reviewed that the dietary phytocompounds such as curcumin, CAPE and sulphoraphane are chemically consist with α, β-unsaturated ketone moiety; therefore, they serve as a potent antioxidant in biological systems (Surh, 2003). Notably, lucidone also structurally architected with α, β-unsaturated ketone moiety. Therefore, we believe that the highly conserved α, β-unsaturated ketone moiety may responsible for the antioxidant and hepatoprotective activity of lucidone. However, further extensive study is highly warranted to disclose this inhibitory mechanism.

In conclusion, lucidone has been received appreciable attention due to the anti-inflammatory activity in both *in vitro* and *in vivo* models (Wang et al., 2008; Kumar and Wang, 2009; Kumar et al., 2010). In the present study, we also found a positive result that lucidone pretreatment protects hepatic cells from acute alcohol-induced oxidative stress. As shown in Fig. 6, the proposed hepatoprotective action of lucidone is most likely to be mediated by the induction of HO-1 *via* Nrf-2 signaling pathway that may provide a pivotal mechanism for its hepatoprotective action.

Conflict of interest statement

The authors(s) declare that there is no conflict of interest.

References

Ambadath, V., Venu, R.G., Madambath, I., 2010. Comparative study of the efficacy of ascorbic acid, quercetin, and thiamine for reversing ethanol-induced toxicity. J. Med. Food. 13, 1485–1489.

Anonymous, 2009. World Health Report: Management of Substance Abuse: Alcohol. World Health Organization, Geneva. <http://www.who.int/topics/alcohol_drinking/en/>.

Artun, B.C., Kusku-Kiraz, Z., Gulluoglu, M., Cevikbas, U., Kocak-Toker, N., Uysal, M., 2010. The effect of carnosine pretreatment on oxidative stress and hepatotoxicity in binge ethanol administered rats. Hum. Exp. Toxicol. 29, 659–665.

Baldi, A., Burra, P., Plebani, M., Salvagnini, M., 2003. Serum malondialdehyde and mitochondrial aspartate aminotransferase activity as markers of chronic alcohol intake and alcoholic liver disease. Ital. J. Gastroenterol. 25, 429–432.

Bao, W., Li, K., Rong, S., Yao, P., Hao, L., Ying, C., Zhang, X., Nussler, A., Liu, L., 2010. Curcumin alleviates ethanol-induced hepatocytes oxidative damage involving heme oxygenase-1 induction. J. Ethnopharmacol. 128, 549–553.

Batey, R.G., Salmond, S.J., Bensoussan, A., 2005. Complementary and alternative medicine in the treatment of chronic liver disease. Curr. Gastroenterol. 7, 63–70.

Braudeau, C., Bouchet, D., Toquest, C., Tesson, L., Menoret, S., Iyer, S., Loboisse, C., Willis, D., Jarry, A., Buelow, R., Anegon, I., Chauveau, C., 2003. Generation of the heme oxygenase-1-transgenic rats. Exp. Biol. Med. 228, 466–471.

Das, S.K., Mukherjee, S., Gupta, G., Rao, D.N., Vasudevan, D.M., 2010. Protective effect of resveratrol and vitamin E against ethanol-induced oxidative damage in mice: biochemical and immunological basis. Ind. J. Biochem. Biophys. 47, 32–37.

Das, S.K., Vasudevan, D.M., 2007. Alcohol-induced oxidative stress. Life Sci. 81, 177–187.

Dambach, D.M., Andrews, B.A., Moulin, F., 2005. New technologies and screening strategies for hepatotoxicity: use of *in vitro* models. Toxicol. Pathol. 33, 17–26.

Davis, R.L., Dertien, J., Syapin, P.J., 2002. Ethanol-induced modulation of inducible nitric oxide synthase activity in human A172 astrocytoma cells. Alcohol. Clin. Exp. Res. 26, 1404–1411.

Deng, X.S., Deitrich, R.A., 2007. Ethanol metabolism and effects: nitric oxide and its interaction. Curr. Clin. Pharmacol. 2, 145–153.

Eklow-Lastbom, L., Moldeus, P., Orvenius, S., 1986. On the mechanisms of glutathione depletion in hepatocytes exposed to morphine and ethylmorphine. Toxicology 42, 13–21.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynoenal malonaldehyde and related aldehyded. Free Radic. Biol. Med. 11, 81–128.

Farombi, E.O., Shrotriya, S., Na, H.K., Kim, S.H., Surh, Y.J., 2008. Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf-2-mediated induction of heme oxygenase-1. Food Chem. Toxicol. 46, 1279–1287.

Fernandez-Checa, J.C., Bellentani, S., Tribelli, C., 2002. Alcohol-induced liver disease: from molecular damage to treatment. Rev. Med. Chile 30, 681–690.

Fernandez-Checa, J.C., Colell, A., Mari, M., Gracia-Ruiz, C., 2005. Ceramide, tumor necrosis factor and alcohol-induced liver disease. Alcohol. Clin. Exp. Res. 29, 151S–157S.

Fotakis, G., Timbrell, J.A., 2006. *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett. 160, 171–177.

Guitierrez-Ruiz, M.C., Quiroz, S.C., Souza, V., Bucio, L., Hernandez, E., Olivares, I.P., Llorente, L., Vargas-Vorackova, F., Kershenobich, D., 1999. Cytokines, growth factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde and LPS. Toxicology 134, 197–207.

Kaur, G., Meena, C., Veeranjaneyulu, A., Agrawal, S.S., 2009. Evaluation of hepatoprotective activity of *Ocumum scantum* in HepG2 cell line. Arch. Pharm. Sci. Res. 1, 25–30.

Khanal, T., Choi, J.H., Hwang, Y.P., Chung, Y.C., Jeong, H.G., 2009. Saponins isolated from the root of *Plantycodon grndiflorum* protect against acute ethanol-induced hepatotoxicity in mice. Food Chem. Toxicol. 47, 2749–2754.

Kirkali, G., Gezer, S., Umur, N., Ozcan, M.A., Tankurt, E., 2000. Nitric oxide in chronic liver disease. Turk. J. Med. Sci. 30, 511–515.

Kumar, K.J.S., Chu, F.H., Hsieh, H.W., Liao, J.W., Li, W.H., Lin, J.C., Shaw, J.F., Wang, S.Y., 2011. Antroquinonol from ethanolic extract of mycelium of *Antrodia cinnamomea* protects hepatic cells from ethanol-induced oxidative stress through Nrf-2 activation. J. Ethnopharmacol. 136, 168–177.

Kumar, K.J.S., Shei, H.W., Wang, S.Y., 2010. Anti-inflammatory effect of lucidone in mice *via* inhibition of NF-κB/MAP kinase pathway. Int. Immunopharmacol. 10, 385–392.

Kumar, K.J.S., Wang, S.Y., 2009. Lucidone inhibits iNOS and COX-2 expression in LPS-induced RAW264.7 murine macrophage cells via NF-κB and MAPKs signaling pathway. Planta Med. 75, 494–500.

Lee, C.H., Park, S.W., Kim, Y.S., Kang, S.S., Kim, J.A., Lee, S.H., Lee, S.M., 2007. Protective mechanism of glycyrrhizin on acute live injury induced by carbon tetrachloride in mice. Biol. Pham. Bull. 30, 1898–1904.

Lee, S.B., Kim, C.Y., Lee, H.J., Yun, J.H., Nho, C.W., 2009. Induction of the phase II detoxification enzyme NQO1 in hepatocarcinoma cells by lignans from the fruit of *Schisandra chinensis* through nuclear accumulation of Nrf2. Planta Med. 75, 1314–1318.

Lin, C.T., Kumar, K.J.S., Tseng, Y.H., Wang, Z.J., Pan, M.Y., Xiao, J.H., Chien, S.C., Wang, S.Y., 2009. Anti-inflammatory activity of flavokawain B from *Alpenia pricei* Hayata. J. Agric. Food Chem. 57, 6060–6065.

Liu, L., Yao, P., Zhang, X., Hao, L., Yang, X., Sun, X., 2004a. Effect of acute ethanol exposure on HO-1 enzyme activity in human primary hepatocytes. Acta Nutr. Sin. 33, 537–539.

Liu, L.G., Yang, H., Zhang, W., Yao, P., Zhang, X.P., Sun, X.F., Nussler, A.K., 2004b. Induction of heme oxygenase-1 in human hepatocytes to protect them from ethanol-induced cytotoxicity. Biomed. Environ. Sci. 17, 315–326.

Liu, S., Hou, W., Yao, P., Zhang, B., Sun, S., Nussler, A.K., Liu, L., 2010. Quercetin protects ethanol-induced oxidative damage in rat primary hepatocytes. Toxicol. In Vitro 24, 516–522.

Maines, M.D., Gibbs, P.E., 2005. 30 some years of heme oxygenase: from a molecular wrecking ball to a mesmerizing trigger of cellular events. Biochem. Biophys. Res. Commun. 338, 568–577.

Majano, P.L., Medina, J., Zubia, I., Sunyer, L., Pezzi, E.L., Rodriguez, A.M., Cabrera, M.L., Otero, R.M., 2004. *N*-acetyl-cysteine modulates inducible nitric oxide synthase gene expression in human hepatoma cells. J. Hepatol. 40, 632–637.

Motohashi, H., Kimura, M., Fujita, R., Inone, A., Pan, X., Takayama, M., Katsuoka, F., Aburatani, H., Bresnik, E.H., Yamamoto, M., 2010. NF-E2 domination over Nrf2 promotes ROS accumulation and megakaryocytic maturation. Blood 115, 677–686.

Nah, H.Y., Lee, W.S., Joo, Y.E., Kim, H.S., Choi, S.K., Rew, J.S., Kim, S.J., Jung, Y.D., Ahn, B.W., Park, J.S., Shin, B.A., 2005. Resveratrol protects HepG2 and Chang liver cells from oxidative stress. Chonnam. Med. J. 41, 243–252.

Neuman, M.G., Koren, G., Tiribelli, C., 1993. *In vitro* assessment of the ethanol-induced hepatotoxicity on HepG2 cell line. Biochem. Biophys. Res. Commun. 197, 932–941.

Oh, H.M., Choi, S.K., Lee, J.M., Lee, S.K., Kim, H.Y., Han, D.C., Kim, H.M., Son, K.H., Kwon, B.M., 2005. Cyclopentenediones, inhibitors of farnesyl protein transferase and anti-tumor compounds, isolated from the fruit of *Lindera erythrocarpa* Makino. Bioorg. Med. Chem. 13, 6182–6187.

Oliva, J., Bardag-Gorce, F., Tillman, B., French, S.W., 2011. Protective effect of quercetin, EGCG, catechin and betaine against oxidative stress induced by ethanol in vitro. Exp. Mol. Pathol. 90, 295–299.

Peng, H.C., Chen, J.R., Chen, Y.L., Yang, S.C., Yang, S.S., 2010. Beta-carotene exhibits antioxidant and anti-apoptotic properties to prevent ethanol-induced cytotoxicity in isolated rat hepatocytes. Phytother. Res. Suppl. 2, S183–189.

Rajakrishanan, V., Menon, V.P., Rajashekaran, K.N., 1998. Protective role of curcumin in ethanol toxicity. Phytother. Res. 12, 55–56.

Song, Z., Deaciuc, I., Song, M., Lee, D.Y., Liu, Y., Ji, X., McClain, C., 2006. Silymarin protects against acute ethanol-induced hepatotoxicity in mice. Alcohol. Clin. Exp. Res. 30, 407–413.

Surh, Y.J., 2003. Cancer chemoprevention with dietary phytochemicals. Nat. Rev. Cancer 3, 768–778.

Wang, S.Y., Lan, X.Y., Xiao, J.H., Yang, J.C., Kao, Y.T., Chang, S.T., 2008. Anti-inflammatory activity of *Lindera erythrocarpa* fruits. Phytother. Res. 22, 213–216.

Yao, P., Nussler, A., Liu, L., Hao, L., Song, F., Schirmeier, A., Nussler, N., 2007. Quercetin protects human Hepatoma cells from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf-2 pathway. J. Hepatol. 47, 253–261.

Zhao, J., Chen, H., Li, Y., 2008. Protective effect of bicyclol on acute alcohol-induced liver injury in mice. Eur. J. Pharmacol. 586, 322–331.