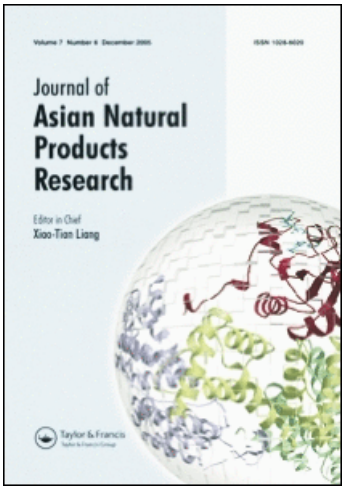

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Involvement of HSP70 in the protection of bicyclol on apoptosis of HepG2 cells intoxicated by d-galactosamine

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Online publication date: 21 April 2010

To cite this Article Bao, Xiu-Qi and Liu, Geng-Tao(2010) 'Involvement of HSP70 in the protection of bicyclol on apoptosis of HepG2 cells intoxicated by d-galactosamine', Journal of Asian Natural Products Research, 12: 4, 313 — 323
To link to this Article: DOI: 10.1080/10286021003769924
URL: http://dx.doi.org/10.1080/10286021003769924

Journal of Asian Natural Products Research
Vol. 12, No. 4, April 2010, 313–323

ORIGINAL ARTICLE

Involvement of HSP70 in the protection of bicyclol on apoptosis of HepG2 cells intoxicated by D-galactosamine

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(*Received 30 December 2009; final version received 11 March 2010*)

Heat shock proteins (HSPs), the best known endogenous factors, play important roles in the cytoprotection and repair of cells and tissues against the harmful effects of stress and insults. In this study, RNAi technology was used to identify whether HSP70 was involved in the protection of bicyclol against D-galactosamine (D-GaIN)-induced apoptosis in HepG2 cells. As a result, bicyclol induced HSP70 in a time- and dose-dependent manner in HepG2 cells. Bicyclol markedly alleviated apoptosis and caspase-3 activity in HepG2 cells intoxicated by D-GaIN. The degradation of inhibitory kappa B, phosphorylation of inhibitory kappa B kinase, nuclear factor kappa B (NF-κB) nuclear translocation, and DNA-binding activity were all inhibited by bicyclol in HepG2 cells intoxicated by D-GaIN. In addition, bicyclol decreased the nitric oxide production and inducible nitric oxide synthase (iNOS) expression. The inhibitory effects of bicyclol on all the above biomarkers were attenuated when the HSP70 gene was silenced accordingly. Our data also showed that MG132 (inhibitor of NF-κB) and NG-nitro-L-arginine methyl ester (inhibitor of iNOS) inhibited hepatocyte apoptosis induced by D-GaIN. These *in vitro* results suggested that HSP70 partially contributed to the hepatoprotection of bicyclol through suppressing the NF-κB–iNOS pathway.

Keywords: bicyclol; heat shock protein; apoptosis; nuclear factor kappa B; inducible nitric oxide synthase

1. Introduction

Bicyclol (**1**) [4,4′-dimethoxy-5,6,5′6′-bis (methylene-dioxy)-2-hydroxymethyl-2′-methoxycarbonyl biphenyl] is a new anti-hepatitis drug (Figure 1), which has been approved to treat chronic viral hepatitis in China. The drug significantly reduces elevated serum alanine aminotransferase and aspartate aminotransferase levels, and also inhibits hepatitis virus B replication to a certain degree in patients suffering from chronic viral hepatitis B [1], while no noticeable side effects have been observed. Most of the protective effects of **1** attribute to its elimination of free radical, maintenance of mitochondrial glutathione redox status, anti-inflammation, and inhibition of fibrogenesis [2]. It is worth noting that preadministration of **1** reduces inducible nitric oxide synthase (iNOS) expression and nuclear factor kappa B (NF-κB)-binding activity [3]. Nevertheless, the detailed molecular mechanism through which **1** regulates iNOS expression is not completely understood.

Heat shock proteins (HSPs) consist of both stress-inducible and constitutive family members. An important feature of HSPs is their role in the cytoprotection and repair of cells and tissues against the

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286021003769924
http://www.informaworld.com

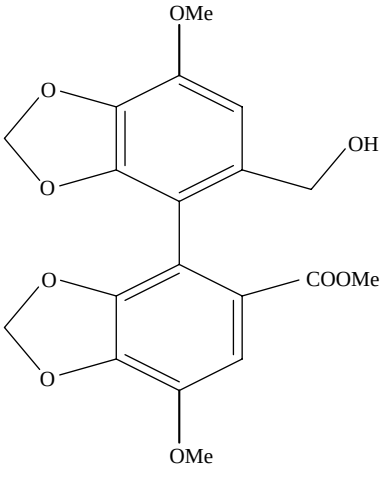

Figure 1. The chemical structure of **1**.

harmful effects of stress and insults. Overexpression of HSP genes is sufficient to protect against otherwise lethal exposures to heat, cytotoxic drugs, toxins, and tumor necrosis factor-α [4]. Studies have shown that HSP70 overexpression confers liver protection, as observed by resistance to acetaminophen-induced toxicity [5,6]. These observations suggest new therapeutic strategies relying upon the development of drugs that are able to increase the expression of HSPs.

Our previous study has identified **1** as a non-toxic HSP inducer, which protects against experimental liver injury caused by acetaminophen [7] and concanavalin A (ConA) [8]. However, the underlying mechanisms of **1**-induced HSPs on the protection against liver injury still need investigation. Thus, we sought to study the stress response induced by **1** in HepG2 cells using RNAi technology. In the current study, we found that HSP70 mediated the hepatocyte-protective roles of **1** in the D-galactosamine (D-GaIN)-induced hepatocyte apoptosis through the inhibition of the NF-κB–iNOS pathway.

2. Results

2.1 Compound 1 induced the expression of HSP70 in HepG2 cells

Since the induction of HSP is a universal stress response against various insults and has been widely shown to have an anti-apoptosis effect, we examined the expression of HSP70, the major member in the HSP family. As shown in Figure 2(A), **1** induced the overexpression of the HSP70 in a time-dependent manner in HepG2 cells. The maximum induction is 2 h (2.14-fold increase, $P < 0.05$), and then gradually declined to the control level at 24 h (Figure 2(A)). The results also showed that **1** induced the expression of HSP70 in a dose-dependent manner both in protein (Figure 2(B)) and mRNA levels (Figure 2(C)). The inducing effect of HSP70 by **1** at 100 μM was more potent than those at 50 and 25 μM.

2.2 Compound 1-induced HSP70 reduced apoptosis of HepG2 cells intoxicated by D-GaIN

As shown in Figure 3(A), the majority of cells in the control group had uniformly stained nuclei after staining with the membrane-permeable DNA-binding dye Hoechst 33258. Exposure to 50 mM D-GaIN for 8 h induced nuclei fragmentation with condensed chromatin and bright staining in morphology of HepG2 cells under fluorescent microscope, indicating the apoptosis. The treatment of **1** at 100 μM prevented the changes in nuclei morphology of HepG2 cells induced by D-GaIN. In addition, when the HSP70 gene was silenced by siRNA, the suppression of apoptosis was inhibited.

Compound **1** also significantly inhibited the activity of caspase-3 caused by D-GaIN in HepG2 cells. When the HSP70 gene was silenced, the activity of caspase-3 enhanced, compared with **1** treated alone (Figure 3(B)). These results suggested that HSP70 was involved in the protection of hepatocyte apoptosis.

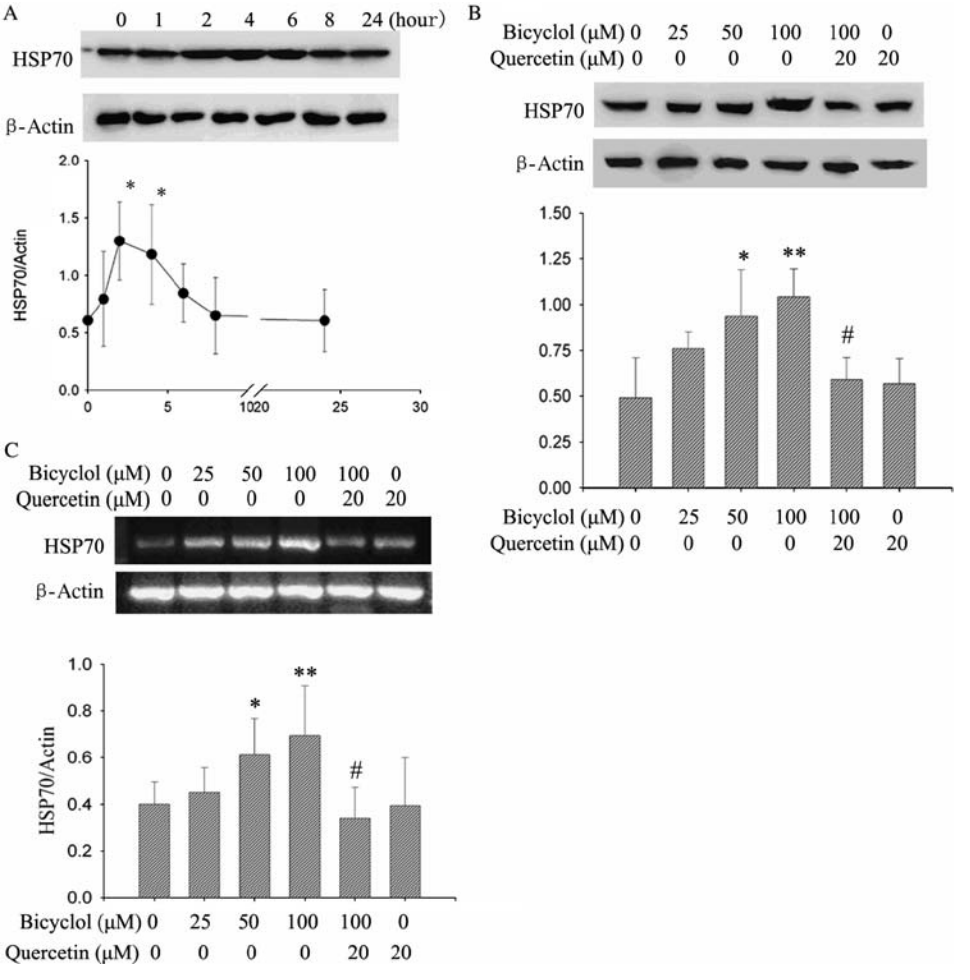

Figure 2. Effect of **1** on the expression of HSP70 in HepG2 cells. (A) Time course of HSP70 induction by **1**. HepG2 cells were treated with 50 μM of **1**. Cells were collected 1, 2, 4, 6, 8, and 24 h later. The HSP70 level was measured by Western blot $^{*}P < 0.05$ vs. 0 h control cells. (B,C) Dose–effect relationship of **1** in inducing HSP70 in HepG2 cells. HepG2 cells were treated with 25, 50, and 100 μM of **1** alone or co-treated with 20 μM of quercetin. Cells were collected 2 h later. The HSP70 level was measured by Western blot and RT-PCR. Data were described as mean ± SD by three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control cells, $^{\#}P < 0.05$ vs. cells treated with 100 μM of **1**.

2.3 NF-κB-mediated hepatocyte apoptosis induced by D-GaIN

To clarify whether hepatocyte apoptosis induced by D-GaIN was mediated by NF-κB, the proteasome inhibitor MG132, which is a well-known NF-κB inhibitor by blocking degradation of inhibitory kappa B (IκB)-α, was employed. MG132 reduced the levels of nitric oxide (NO) in HepG2 cells after D-GaIN was added, and **1** decreased the levels of NO challenged with D-GaIN (Figure 4(A)). MG132 also decreased caspase-3 activity in D-GaIN-challenged HepG2 cells (Figure 4(B)). The data indicated that the increased levels of NO induced by D-GaIN were NF-κB-mediated, and **1** decreased the levels of NO and caspase-3 through the direct inhibition of NF-κB activity.

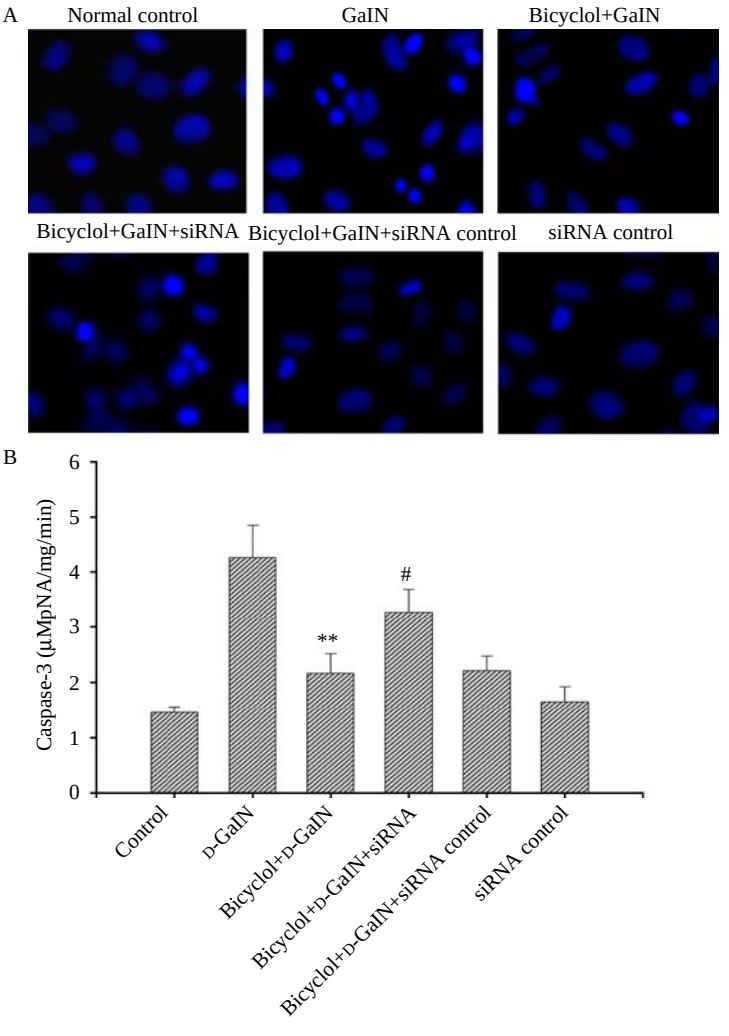

Figure 3. Compound **1**-induced HSP70 inhibited apoptosis of HepG2 cells intoxicated with D-GaIN. The cells were pretreated with 100 μM of **1** alone or transfected with HSP70 siRNA simultaneously, and then they were stimulated 50 mM of D-GaIN, and harvested 8 h later. (A) Hoechst 33258 staining of DNA in HepG2 cells. (B) Caspase-3 activity in HepG2 cells. $^{**}P < 0.01$ vs. D-GaIN-treated cells, $^{\#}P < 0.05$ vs. **1** + D-GaIN-treated cells. Each bar represents the mean ± SD of five separate experiments.

2.4 Inhibitory effect of 1-induced HSP70 on the NF-κB pathway in HepG2 cells intoxicated by D-GaIN

IκB inhibits the transcriptional activity of NF-κB by preventing the nuclear translocation of NF-κB in the cytoplasm. Here, we showed that after HepG2 cells were treated with D-GaIN, the degradation of IκB was observed. Pretreatment of **1** at a concentration of 100 μM decreased IκB degradation challenged by D-GaIN. RNAi HSP70 significantly decreased the inhibitory effect of **1** on IκB degradation (Figure 5(A)). Compound **1** also inhibited the activity of phosphorylated inhibitory kappa B kinase (IKK) activated by D-GaIN. When the HSP70 gene was silenced, the inhibitory

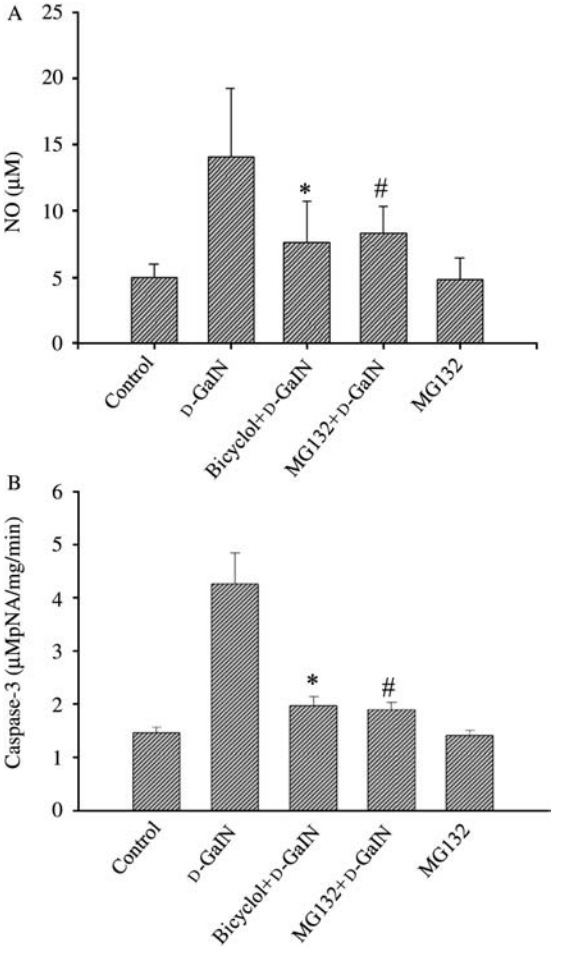

Figure 4. Hepatocyte apoptosis induced by D-GalN was NF-κB-mediated. HepG2 cells were treated with 20 μM of MG132, and then they were stimulated with 50 mM of D-GalN, and harvested 8 h later. (A) NO production. (B) Caspase-3 activity. $^{*,\#}P < 0.05$ vs. D-GalN-treated cells. Each bar represents the mean ± SD of five separate experiments.

effect of **1** on phosphorylated IKK was attenuated (Figure 5(B)).

Next, we studied the effect of **1**-induced HSP70 on NF-κB activation in D-GalN-treated HepG2 cells. Pretreatment of **1** inhibited nuclear translocation of the NF-κB p65 subunit and NF-κB-DNA-binding activity induced by D-GalN. However, when HSP70 synthesis was inhibited by RNAi, the suppression of nuclear translocation of the NF-κB p65 subunit and NF-κB-DNA-binding activity of **1** was abrogated. The results indicated that **1**-induced HSP70 might be involved in the suppression of the NF-κB pathway activation (Figure 5(C)).

2.5 Regulation of iNOS by 1-induced HSP70 in HepG2 cells challenged by D-GalN

NF-κB is the most relevant transcription factor that regulates iNOS expression in hepatocytes. The results showed that the expression of iNOS in HepG2 cells treated with D-GalN was inhibited by adding

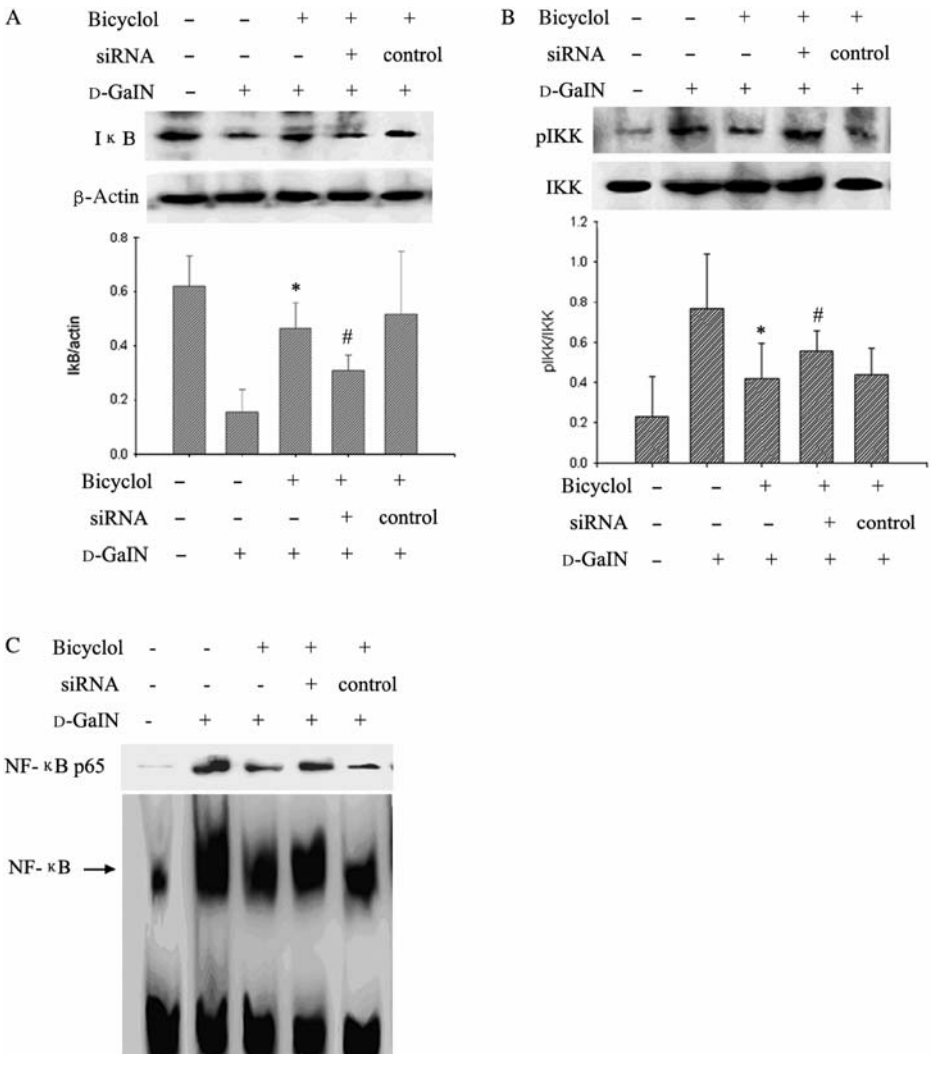

Figure 5. Inhibitory effect of **1**-induced HSP70 on NF-κB activation in HepG2 cells intoxicated by D-GalN. The cells were pretreated with 100 μM of **1** alone or transfected with HSP70 siRNA simultaneously, and then they were stimulated with 50 mM of D-GalN, and harvested 8 h later. (A) IκB degradation, (B) (p)IKK expression and (C) NF-κB p65 translocation and NF-κB-DNA binding activity in HepG2 cells. Data were described as mean ± SD by three separate experiments. $^{*}P < 0.05$ vs. D-GalN-treated cells; $^{\#}P < 0.05$ vs. **1** + D-GalN-treated cells.

MG132 (Figure 6(A)), indicating that the expression of iNOS was under the control of NF-κB.

Compound **1**-induced HSP70 on the iNOS expression in HepG2 cells was examined. The results showed that **1** decreased the expression of iNOS in HepG2 cells challenged by D-GalN. Silencing the HSP70 gene inhibited the effect of **1** on the iNOS expression. The expression of iNOS was blocked by NG-nitro-L-arginine methyl ester (L-NAME; Figure 6(B)).

To study whether the iNOS expression contributed to the apoptosis of the hepatocyte, L-NAME was used to block the expression of iNOS. As a result, adding

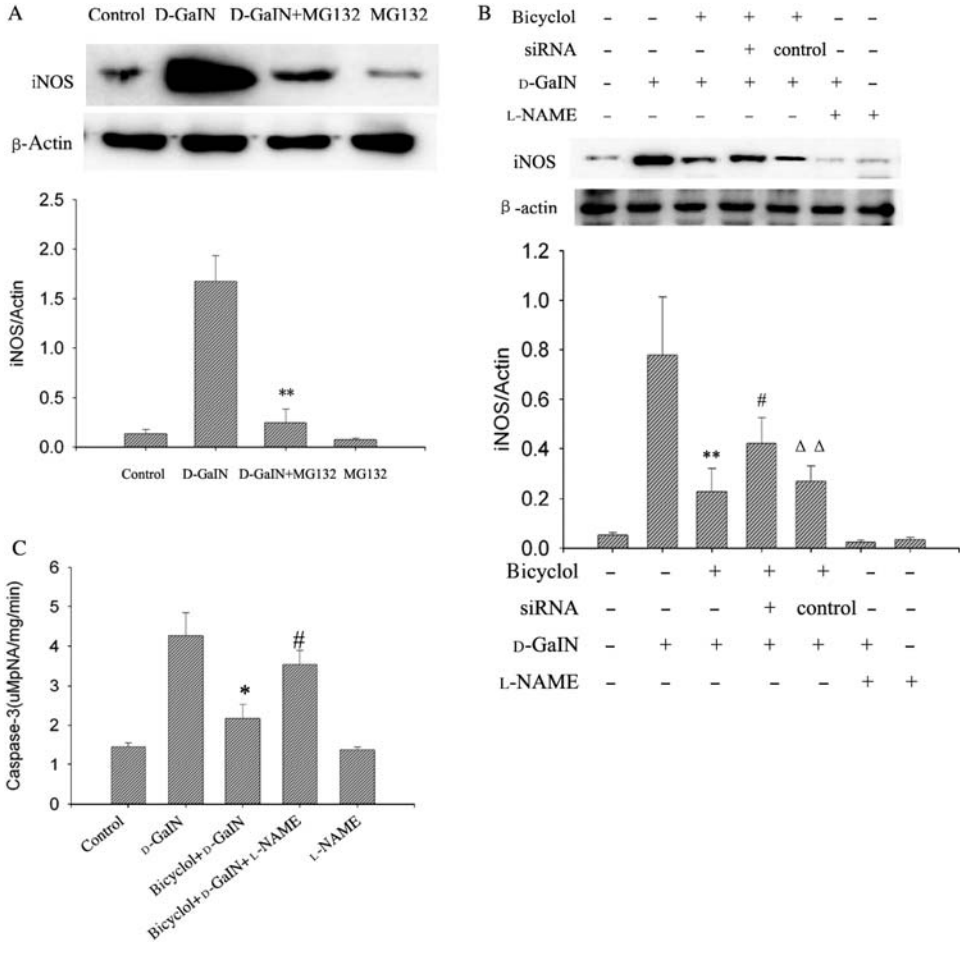

Figure 6. Effect of **1**-induced HSP70 on iNOS in HepG2 cells challenged by D-GaIN. Cells were treated as described in Section 4. (A) MG132 on iNOS expression. $^{**}P < 0.01$ vs. D-GaIN-treated cells. (B) Compound **1**-induced HSP70 on iNOS expression. $^{**}P < 0.01$ vs. D-GaIN-treated cells; $^{\#}P < 0.05$ vs. **1** + D-GaIN-treated cells; $^{\Delta}P < 0.05$ vs. D-GaIN-treated cells. (C) L-NAME on caspase-3 activity. $^{*}P < 0.05$ vs. D-GaIN-treated cells; $^{\#}P < 0.05$ vs. **1** + D-GaIN-treated cells. All experiments are repeated at least three times.

L-NAME in HepG2 cells significantly decreased the inhibitory effect of **1** on caspase-3 activity challenged by D-GaIN (Figure 6(C)). The results suggested that apoptosis caused by D-GaIN was NF-κB–iNOS-mediated, which was suppressed by **1**-induced HSP70 in the hepatocyte.

3. Discussion

In the present study, we showed that **1** inhibited the activation of the NF-κB–iNOS pathway in HepG2 cells intoxicated by D-GaIN. We also found that **1** can induce HSP70 overexpression, and when the expression of the HSP70 gene was silenced, the inhibition of NF-κB and iNOS was attenuated correspondingly. Our study suggested a possible molecular mechanism underlying **1**-induced cell survival in D-GaIN-challenged hepatocytes, i.e. **1**-induced HSP70 may suppress NF-κB activity and NO production, thus consequently inhibit hepatocyte apoptosis. This assumption is supported by the following

evidences. First, NF-κB activation occurred in response to D-GaIN stimulation and NO was inhibited by MG132, an inhibitor of NF-κB. These data indicated that D-GaIN-induced hepatocyte apoptosis was mediated by NF-κB in HepG2 cells. In addition, studies have shown that HSP70 protects liver injury through inhibiting NF-κB activation and NO production [9]. Silencing the HSP70 gene attenuated the inhibitory effect of **1** on NF-κB activation and NO production in HepG2 cells. So, our study provided evidence for elucidating that HSP70 may contribute to the hepatoprotective effect of **1** through suppressing the NF-κB–iNOS pathway.

NF-κB is responsible for regulating the transcription of a large number of genes involved in inflammation, immunity, cancer, and apoptosis [10]. When NF-κB was activated, it then translocated to the nucleus, where it binds to various target genes [11], one of which is iNOS [12]. iNOS produces NO, which regulates apoptosis. Accordingly, prevention of NF-κB activation inhibits the iNOS expression [13,14]. NF-κB activation can be inhibited by anti-inflammatory agents and antioxidant inhibitors [15]. As an anti-hepatitis drug, **1** has both anti-inflammatory and antioxidant effects [2]. In this study, the inhibitory effect of **1** on NF-κB has been verified in HepG2 cells challenged by D-GaIN.

Recent studies demonstrated that HSP induction inhibited signal transduction pathways including NF-κB [16]. As a molecular chaperone, HSP70 could prevent the nuclear translocation of NF-κB, thereby inhibiting iNOS induction [17]. Consistent with previous study that induction of HSPs by **1** suppressed NF-κB activation on ConA-induced mouse hepatitis [7], the present result verified that HSP70 conferred to the inhibition of NF-κB and its target gene iNOS using RNAi technology. This *in vitro* study combined *in vivo* study indicating that HSPs may be one of the drug targets of **1** in protecting against liver injury.

NO plays a key role during the induction of liver injury [18]. In this sense, NO has been shown to mediate [19] or prevent [20] cell death in rat hepatocytes. The induction of cell death by D-GaIN was related to an increase in iNOS expression and NO production by hepatotoxins in hepatocytes [21]. In addition, the inhibition of iNOS reduced D-GaIN-induced apoptosis in cultured hepatocytes [22]. Our finding suggested that iNOS played an important role in hepatocyte apoptosis. Compound **1** pretreatment protected against hepatocyte apoptosis and decreased caspase-3 activity. Furthermore, silencing of the HSP70 gene markedly attenuated the protective actions of **1** in D-GaIN-treated HepG2 cells. We speculated that **1** blocked NF-κB activation, subsequently inhibited the transcription of NO, the target genes of the NF-κB, and finally hepatocytes were protected from D-GaIN-induced hepatic injury.

In conclusion, our present study demonstrated that HSP70 may contribute to the hepatoprotection of **1** through suppressing the NF-κB–iNOS pathway in HepG2 cells. Our finding may provide new therapeutic strategies relying upon the development of drugs that are able to increase the expression of HSPs.

4. Materials and methods

4.1 Cell culture and transfection

Human hepatoma HepG2 cells were maintained in minimal essential medium with Earle's salts, 10% (v/v) heat-inactivated fetal bovine serum, in a humidified incubator under 5% CO_2/95% air at 37°C.

Small interfering HSPA4 dsRNA constructs generated by Invitrogen Life Technologies (Carlsbad, CA, USA) were cloned into pcDNA6.2-GW/EmGFP-miR. HepG2 cells were plated for 24 h and grew to 50–80% confluency prior to transfection. Transfection was performed using

Lipofectamine LTX transfection reagent and Plus reagent (Invitrogen) according to the manufacturer's instructions. The transfection medium was removed 12 h later.

4.2 Treatment of HepG2 cells

HepG2 cells were grown in a 25 cm^2 culture flask at 5×10^5 cells/flask. Fifty micromolar of **1** was used in the time-course study of the inducing effect on HSP70. In addition, 25, 50, and 100 μM of **1** were treated to cells for the dose–effect relationship study. To investigate the protective effect of **1** on HepG2 cells challenged by D-GaIN and the underlying mechanisms, the cells were transiently transfected with HSP70 siRNA, and then treated with 100 μM of **1** 12 h later. The cells were then stimulated with 50 mM of GaIN after 2 h, and harvested 8 h later. L-NAME (1 mM) and MG132 (20 μM) were treated to the cells to block the expression of iNOS and NF-κB, respectively.

4.3 Hoechst 33258 staining

HepG2 cells were grown in 24-microwell plates and treated as described in Section 4. To observe cells undergoing apoptosis, Hoechst 33258 staining was performed according to the kit instruction (Beyotime Institute Biotechnology, Haimen, China). Cells were counted and examined by fluorescence microscopy at 480 nm (Eclipse TE300; Nikon, Tokyo, Japan). At minimum, 500 cells were counted from more than five random microscopic fields by two observers.

4.4 NO determination

The production of NO was determined by assaying the concentration of nitrite in the culture supernatant. Briefly, 100 μl of the culture supernatant was incubated with 100 μl Griess reagent (a 1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine in distilled water) at room temperature for 20 min. Sodium nitrite was used to generate a standard curve. The OD value of the samples at 550 nm was measured.

4.5 Caspase-3 activity determination

Caspase-3 activity was determined according to the manufacturer's protocol (Sigma, St Louis, MO, USA). In brief, HepG2 cell lysates were prepared by lysis buffer (25 mM HEPES, pH 7.4, 2.5 mM CHAPS, 2.5 mM dithiothreitol). Caspase-3 activity was determined by monitoring proteolysis of the colorimetric substrates. Ac-DEVD-*p*-nitroaniline was used as the colorimetric *p*-nitroaniline-linked substrate. The whole-cell lysate was added to a buffer containing 200 μM substrate. After 1.5 h of incubation, the cleavage of the peptide by the caspase was quantified spectrophotometrically at 405 nm in a 96-well plate. The unit of the optical density was converted to nanomoles of *p*-nitroaniline using a standard curve generated with free *p*-nitroaniline.

4.6 Reverse transcription-polymerase chain reaction assay

Total RNA was isolated from cells using the Trizol reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using One-Step RT-PCR Kit (Promega, Fitchburg, WI, USA). The reaction mixture contains 10 μl AMV/Tfl reaction buffer, 0.2 mM dNTP, 1 μM of each primer, 1 mM $MgSO_4$, 0.1 U/μl AMV reverse transcriptase, Tfl DNA polymerase, and 2 μg RNA template. The reaction was heated at 45°C for 45 min for reverse transcription, and at 94°C for 2 min for AMV RT inactivation and RNA/cDNA/primer denaturation for 40 cycles. Denaturation, annealing, and extension steps for detecting HSP70 transcripts were carried out at 94°C for 30 s, at 59°C for 1 min, and at 68°C for 2 min, respectively. The final extension

was at 68°C for 7 min. The following primers used in the PCRs were synthesized by Shanghai Sangon Biological Engineering Technology & Services Company (Shanghai, China): HSP70-forward 5′-GCGACCTGAACAAGAGCATC-3′ and reverse 5′-GAGCTTGCCCTTGAGAC CC-3′, which leads to a 617-bp product. Amplified products were separated on a 1% agarose gel in TBE buffer (45 mM Tris borate, 1 mM EDTA). RT-PCR bands were photographed with a Kodak Gel Logic 100 Imaging System (Life Technologies Inc., Eastman Kodak Co., New Haven, CT, USA) and the density of the bands was determined using Gel-Pro Analyzer 4.0 software.

4.7 Western blot analysis

Cells were lysed in a non-denaturing lysis buffer (Applygen Technologies Inc., Beijing, China). Thirty micrograms of sample proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 10% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in 5% skim milk-TBS-T (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) at 4°C overnight. Blots were probed with antibodies against HSP70, IκB-α, NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), IKK-α, and iNOS (Cell Signaling, Beverly, MA, USA) in 5% skim milk-TBS-T for 2 h at room temperature, and then incubated with the horseradish peroxidase-conjugated secondary antibody in skim milk-TBS-T for 2 h at room temperature. The blot was developed with LAS3000 chemiluminescence system (Fujifilm, Tokyo, Japan) and the density of the bands was determined using the Gel-Pro Analyzer 4.0 software.

4.8 Statistical analysis

Data were expressed as means ± SD. Changes in different assays were analyzed by ANOVA followed by the Tukey–Kramer test as the *post hoc* test. $P < 0.05$ was considered to be statistically significant.

Acknowledgements

This work was financially supported by grants from the Chinese Ministry of Science and Technology (96-901-01-45) and from the Chinese Medical Board in New York (93-582).

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