

Induction of Overexpression of the 27- and 70-kDa Heat Shock Proteins by Bicyclol Attenuates Concanavalin A-Induced Liver Injury through Suppression of Nuclear Factor- κ B in Mice

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ABSTRACT

Heat shock proteins (HSPs) are molecular chaperones critical for cell survival under adverse environmental conditions and for normal cellular homeostasis. Bicyclol, a novel antihepatitis drug, has been shown to protect against liver injury in animals. However, it is unclear how bicyclol protects against liver injury. We recently found that bicyclol is an inducer of HSPs. We wondered whether bicyclol regulated the expression of HSPs to produce a liver protection in vivo. Thus, this study was designed to address these questions using a mouse model with concanavalin A (ConA)-induced liver injury. Oral administration of bicyclol markedly alleviated ConA-caused liver injury in mice as indicated by the reduction of serum aminotransferases, liver necrosis, and the release of cytochrome c and apoptosis-

inducing factor from mitochondria and hepatic DNA fragmentation. Correlated with this, bicyclol induced the increase of mRNA and protein levels of hepatic 27- and 70-kDa HSPs (HSP27 and HSP70) in the mice. Correspondingly, the elevated HSP27 and HSP70 suppressed inhibitor κ B degradation and nuclear factor κ B (NF- κ B) activation that were caused by ConA. The protective effects of bicyclol on ConA-induced mouse liver injury were markedly attenuated by quercetin, an inhibitor of HSPs synthesis. Our results suggest that the antihepatitis drug bicyclol may protect against liver injury by inducing the expression of hepatic HSP27 and HSP70 and consequently inhibit the transcription factor NF- κ B-mediated apoptosis and necrosis in liver tissue.

Heat shock proteins (HSPs) play an important role in cellular homeostasis during normal cell growth and in response to detrimental environmental stresses (Hartl, 1996; Fink, 1999). Among several members of the HSPs family, stress-inducible HSP27 and HSP70 are most intensively studied for their functions in protecting cells and tissues from injury caused by a variety of physiological and pathological agents, such as interleukin-1 (IL-1) (Reilly et al., 2007), tumor necrosis factor- α (TNF- α) (Carlson et al., 2007), and ischemia reperfusion (Kuboki et al., 2007). Studies using cell and animal models have shown that HSPs can be induced at the protein and mRNA levels to promote cell growth and protect tissues from injury (Kültz, 2005). Thus, an enhanced expression of HSPs in cell response to stresses is an important defense mechanism to tissue injury.

In addition to environmental stress, HSPs can also be induced by some pharmacological drugs. For example, geranylgeranyl acetone (Nishida et al., 2006) and dibutylryl cyclic adenosine monophosphate (Takano et al., 1998) can induce the level of HSP70 and increase liver resistance to the damage caused by hepatotoxic compounds or TNF- α . Thus, identifying drugs that are able to enhance HSPs expression and promote cell survival would be beneficial for clinical therapy of chemical-caused liver injury. Previous studies in our laboratory had identified bicyclol [4, 4'-dimethoxy-5, 6, 5'6'-bis(methylene-dioxy)-2-hydroxymethyl-2'-methoxycarbonyl biphenyl] as a new antihepatitis drug. This drug has been used for treatment of patients with chronic viral hepatitis B in China since 2004. It markedly reduced the elevated serum levels of ALT and AST to the normal limits by 50% and also inhibited hepatitis viruses B and C replication by approximately 20% in patients with hepatitis viruses B and C (Yao et al., 2002). Bicyclol also inhibited the replication of hepatitis virus in duck viral hepatitis and a HepG2.2.15 cell line (Liu, 2001). Moreover, bicyclol has protective action against exper-

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ABBREVIATIONS: HSPs, heat shock proteins; HSP27, 27-kDa HSP; HSP70, 70-kDa HSP; TNF- α , tumor necrosis factor- α ; IL, interleukin; ConA, concanavalin A; RT-PCR, reverse transcription-polymerase chain reaction; HSF1, heat shock factor-1; AIF, apoptosis-inducing factor; HSE, heat shock element; NF- κ B, nuclear factor- κ B; I κ B, inhibitor κ B; IKK, inhibitor κ B kinase; TBS-T, Tris-buffered saline/Tween 20; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal.

imental liver injury. For example, bicyclol reduced mouse liver injury induced by CCl_4 (Liu et al., 2005), acetaminophen (Li et al., 2001), and concanavalin A (ConA) (Zhao and Liu, 2001; Li and Liu, 2004). It is noteworthy that few noticeable side effects have been reported in the clinical application of bicyclol. However, the molecular mechanism underlying this action of bicyclol remains unclear. Our recent study found that bicyclol alone can significantly induce the expression hepatic HSP27 and HSP70 in normal mice, and it can also markedly reduce acetaminophen-induced liver injury through its induction of HSPs (Bao and Liu, 2008). In an *in vitro* study using HepG2 cells, we observed that bicyclol alone can enhance the promoter activities of both HSP27 and HSP70 (unpublished data), indicating that bicyclol induces hepatic HSP27 and HSP70 expression through activating transcription of HSPs genes. Therefore, it is very interesting to study whether bicyclol may protect the liver from chemical-caused injury through HSP-mediated cellular mechanisms.

In an attempt to address these questions, we carried out a set of *in vivo* experiments using a ConA-induced liver injury mouse model. In this animal model system, intravenous injection of ConA can induce hepatitis with manifests of elevated serum ALT levels, T cell infiltration as detected by histological analysis, massive granulocyte accumulation, and hepatocyte apoptosis and necrosis (Tiegs et al., 1992). In the current study, using this model, we found that bicyclol increases the expression of HSP27 and HSP70 in the ConA-treated liver tissues, whereas the enhanced expression of HSP27 and HSP70 mediates the liver protective roles of bicyclol in the ConA-induced liver injury through inhibition of ConA-stimulated inflammatory responses.

Materials and Methods

Animals. Male Kunming mice weighing 20 to 22 g were supplied by the Animal Center of Chinese Academy of Medical Sciences (Beijing, China). Mice were maintained in a 12-h light/dark cycle at 24°C in a room containing relative humidity of 60% and received food and water *ad libitum*. All experimental procedures were performed in accordance with the guidelines of Beijing Municipal Ethic Committee for the Care and Use of Laboratory Animals.

Treatment of Animals. Bicyclol, a white powder with 99% purity, was kindly provided by Beijing Union Pharmaceutical Manufactory (Beijing, China). Mice were orally administered bicyclol 200 mg/kg (dissolved in polyethylene glycol 400) for the study of time course effect on hepatic HSPs. Bicyclol 100, 200, and 300 mg/kg was administered for dose-dependent experiments. Control mice received the same volume of polyethylene glycol 400 only as that for the experimental mice. The protocol for bicyclol treatment was 8:00 AM and 6:00 PM on the first day and 8:00 AM on the second day of the experiment. Mice were sacrificed at the indicated time points after the last administration of bicyclol. Quercetin (Sigma, St. Louis, MO) was suspended in pathogen-free saline and administered orally to mice at a dosage of 200 mg/kg in association with bicyclol. The volume of each compound and vehicle was 10 ml/kg.

ConA 25 mg/kg (Sigma) dissolved in pathogen-free saline was injected intravenously to mice 1 h after the last administration of bicyclol. Control mice were injected with the same volume of saline. Mice were sacrificed 6 h after ConA injection for assays as described below. In the case of NF- κ B inhibition, MG132 2 mg/kg (Sigma) dissolved in dimethyl sulfoxide was injected intraperitoneally to mice 1 h before ConA injection.

Reverse Transcription-Polymerase Chain Reaction Assay. Total RNA was isolated from liver tissues using TRIzol reagent

(Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using One-Step RT-PCR Kit (Promega, Madison, WI). The reaction mixture contained 10 μ l of AMV/Tfl reaction buffer (Promega), 0.2 mM dNTP, 1 μ M concentration of each primer, 1 mM MgSO_4 , 0.1 μ l/ μ l AMV reverse transcriptase and Tfl DNA polymerase, and 2 μ g of RNA template. The reaction was heated at 45°C for 45 min for reverse transcription and 94°C for 2 min for AMV RT inactivation and RNA/cDNA/primer denaturation for 40 cycles. Denaturation, annealing, and extension steps for detecting HSP27 transcripts were 94°C for 30 s, 55°C for 1 min, and 68°C for 2 min, respectively. The final extension was at 68°C for 7 min. The same protocol was also used for HSP70, except the annealing temperature was set 59°C for 1 min. The following primers used in the PCR reactions were synthesized by Shanghai Sangon Biological Engineering Technology and Services Company (Shanghai, China): HSP27: forward, 5'-CCCACCCTCTATCACGGCTAC-3'; reverse, 5'-GGGCTCAACTCTGGCTATCTC-3', which leads to a 426-bp product; and HSP70: forward, 5'-GCGACCTGAACAAGAGCATC-3'; reverse, 5'-GAGCTTGCCCTTGAGACCC-3', which leads to a 617-bp product. Amplified products were separated on a 1% agarose gel in buffer containing 45 mM Tris borate and 1 mM EDTA. RT-PCR bands were photographed with a Kodak Gel Logic 100 Imaging System (Carestream Health, Rochester, NY), and the density of the bands was determined using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Bethesda, MD).

Western Blot Analysis. Mouse liver tissues were homogenized in nondenaturing lysis buffer (Applygen Technologies Inc., Beijing, China). Sample proteins (30 μ g) were separated by SDS-polyacrylamide gel electrophoresis on 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, and 0.1% Tween 20) at 4°C overnight. Blots were probed with antibodies against HSP27, HSP70, inhibitor κ B- α (I κ B- α), NF- κ B p65, cytochrome *c*, apoptosis-inducing factor (AIF; Santa Cruz Biotechnology, Santa Cruz, CA), and inhibitor κ B kinase- α (IKK- α ; Cell Signaling Technology, Danvers, MA) 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 Gel-Pro Analyzer 4.0 software.

Electrophoretic Mobility Shift Assay. Liver nuclear extracts for electrophoretic mobility shift assay were prepared using nuclear-cytosol extraction kit (Applygen Technologies Inc.). Annealed double-stranded heat shock element (HSE) oligonucleotides (5'-AGA CGC GAA ACT GCT GGA AGA TTC CGT GCC-3') and NF- κ B consensus oligonucleotides (5'-GCC TGG GAA AGT CCC CTC AAC T-3') labeled with biotin were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Electrophoretic mobility shift assay kit (Pierce, Rockford, IL) was used to perform the reaction. The binding reaction (20 μ l in total) consists of 10 μ g of protein extracts, 20 fmol of biotin labeled DNA, 2.5% glycerol, 5 mM MgCl_2 , 50 ng/ μ l poly(dI · dC), and incubated for 20 min at room temperature. DNA protein complexes were resolved by electrophoresis on a 6% polyacrylamide gel at 4°C in 0.5 \times buffer containing 45 mM Tris borate and 1 mM EDTA and transferred to a nylon membrane. Then the membrane was detected with the enhanced LAS3000 chemiluminescence system.

Coimmunoprecipitation. Mouse livers were lysed in nondenaturing lysis buffer (Applygen Technologies Inc.). The coimmunoprecipitation assay was performed following the protocol of coimmunoprecipitation kit (Pierce). In brief, 50 μ g of the purified HSP27 or HSP70 antibody was immobilized in 100 μ l, 50% antibody coupling gel. Protein extracts (300 μ g) were incubated with gentle end-over-end mixing for 2 h at room temperature. Immunoprecipitated complexes were eluted thrice with 50 μ l of elution buffer, boiled and separated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, incubated with IKK- α or

I κ B- α antibodies, and detected with the enhanced LAS3000 chemiluminescence system.

ALT/AST Determination. The serum level of ALT and AST was determined using commercial kits (Beijing BHKT Clinical Reagent Co., Ltd., Beijing, China).

Liver Histopathology. For liver histopathological examination, mouse livers were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The specimen was examined under a Nikon E600 digital camera-equipped microscope (Nikon, Tokyo, Japan) and was analyzed using Image Pro-Plus 7200 software.

DNA Fragmentation Assay. Mouse liver DNA was extracted using apoptotic DNA ladder kit (Appligen Technologies Inc.). The extracts were dissolved in distilled water and separated by 1% agarose gel electrophoresis.

Cytokine Assay. The levels of serum TNF- α and IL-2 were determined by enzyme-linked immunosorbent assay following the protocol of enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA).

Statistical Analysis. Data were expressed as means \pm S.D. Changes in different assays were analyzed by analysis of variance followed by the Tukey-Kramer test as the post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Bicyclol Induces the Overexpression of Hepatic HSP27 and HSP70 in ConA-Injected Mice. To determine whether bicyclol induces the expression of hepatic HSP27 and HSP70 in ConA-injected mice, we first conducted a time-course experiment. Mice livers were collected at 1, 2, 4, 6, 8, and 24 h after ConA injection. As shown in Fig. 1A, without bicyclol treatment, ConA increased the levels of both HSP70 and HSP27, but the former was peaked earlier (2 h with 1.3-fold increase, $P < 0.05$) than the latter (4 h with 0.8-fold increase, $P < 0.05$). It is noteworthy that preadministration of bicyclol (200 mg/kg three times within 24 h) further enhanced the accumulation of both hepatic HSP27 and HSP70 proteins in ConA-injected mice (Fig. 1B). The level of HSP27 was clearly increased by 2.0-fold in contrast to that at 0 h ($P < 0.01$). The same response was also observed in HSP70, because its level increased by 2.8-fold within 2 h ($P < 0.01$) and remained high until 4 h and then gradually decreased to a level equivalent to that for the control at 24 h (Fig. 1B). Thus, our results show that bicyclol by oral administration can markedly elevate the level of HSP27 and HSP70 in ConA-injected mouse livers.

Dose-Dependent Effect of Bicyclol on Hepatic HSP27 and HSP70 Levels in ConA-Injected Mice. As mentioned above, the increase of hepatic HSP27 and HSP70 expression varied with time after bicyclol (200 mg/kg) administration in ConA-injected mice. To test whether this induction is dose-dependent, mice were administered bicyclol 100, 200, and 300 mg/kg. Administration of bicyclol 100 (HSP27: 1.2-fold increase, $P < 0.05$; HSP70: 1.1-fold increase, $P < 0.05$), 200 (HSP27: 1.86-fold increase, $P < 0.05$; HSP70: 1.92-fold increase, $P < 0.01$), and 300 mg/kg (HSP27: 3.6-fold increase, $P < 0.01$; HSP70: 3.05-fold increase, $P < 0.01$) thrice within 24 h induced hepatic HSP27 and HSP70 accumulation in a dose-dependent manner in ConA-treated mice. The inducing effect of bicyclol 300 mg/kg on HSP27 and HSP70 expression was more potent than those of 100 and 200 mg/kg. This inductive effect of bicyclol on HSP27 (decreased 50%, $P < 0.05$) and HSP70 (decreased 67%, $P < 0.01$) was attenuated

by coadministration of quercetin, an inhibitor of HSPs biosynthesis (Fig. 2A).

We next investigated whether bicyclol pretreatment could also affect the expression of HSP27 and HSP70 genes. RT-PCR analysis showed that bicyclol 200 and 300 mg/kg up-regulated hepatic HSP27 (bicyclol 200 mg/kg: approximately 1-fold increase, $P < 0.05$; bicyclol 300 mg/kg: approximately 1.56-fold increase, $P < 0.05$) and HSP70 (bicyclol 200 mg/kg: approximately 1.65-fold increase, $P < 0.05$; bicyclol 300 mg/kg: approximately 3.2-fold increase, $P < 0.01$) mRNA expression in mice injected with ConA. The induction of HSP27 and HSP70 mRNA by bicyclol 300 mg/kg was highly significant.

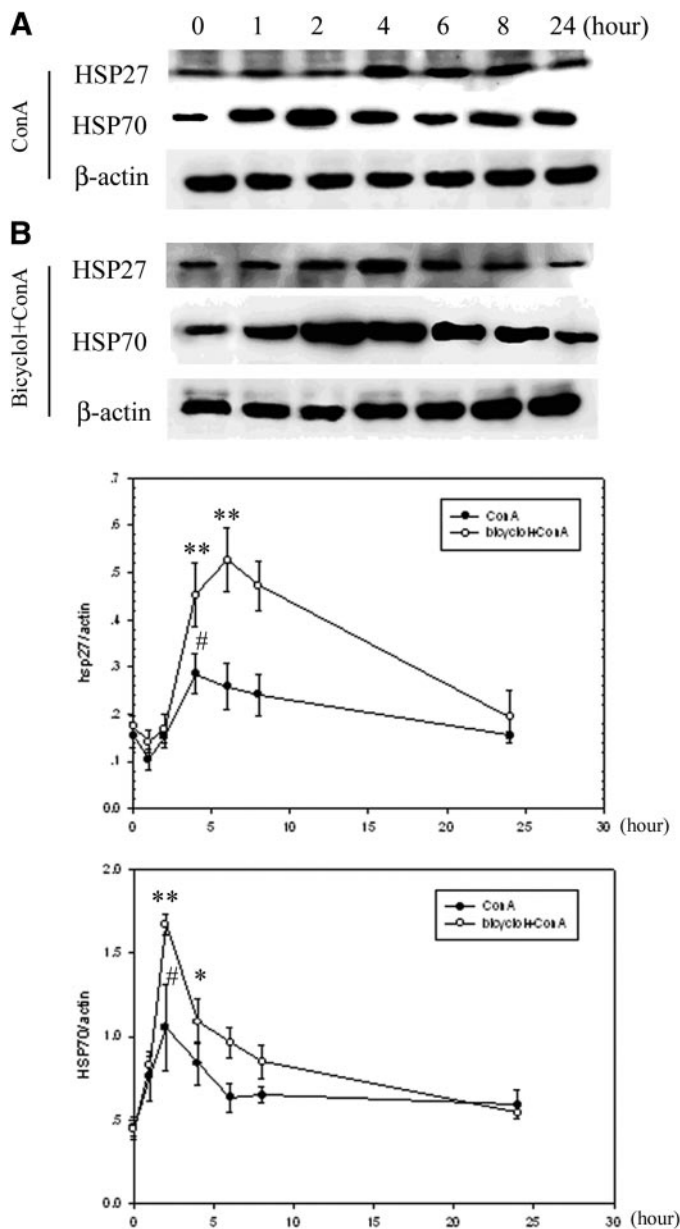


Fig. 1. Time course of ConA (A) and bicyclol plus ConA (B) in inducing hepatic HSP27 and HSP70 expression in mice. Bicyclol 200 mg/kg was given to mice three times in 24 h as described under *Materials and Methods*. The mice were intravenously injected a dose of ConA 25 mg/kg 1 h after the last administration of bicyclol. The liver proteins from mice of different groups were subjected to Western blot analysis with antibodies against HSP27 and HSP70. The blots are representative of three independent experiments. * and #, $P < 0.05$; ** and ##, $P < 0.01$ versus 0-h group, respectively.

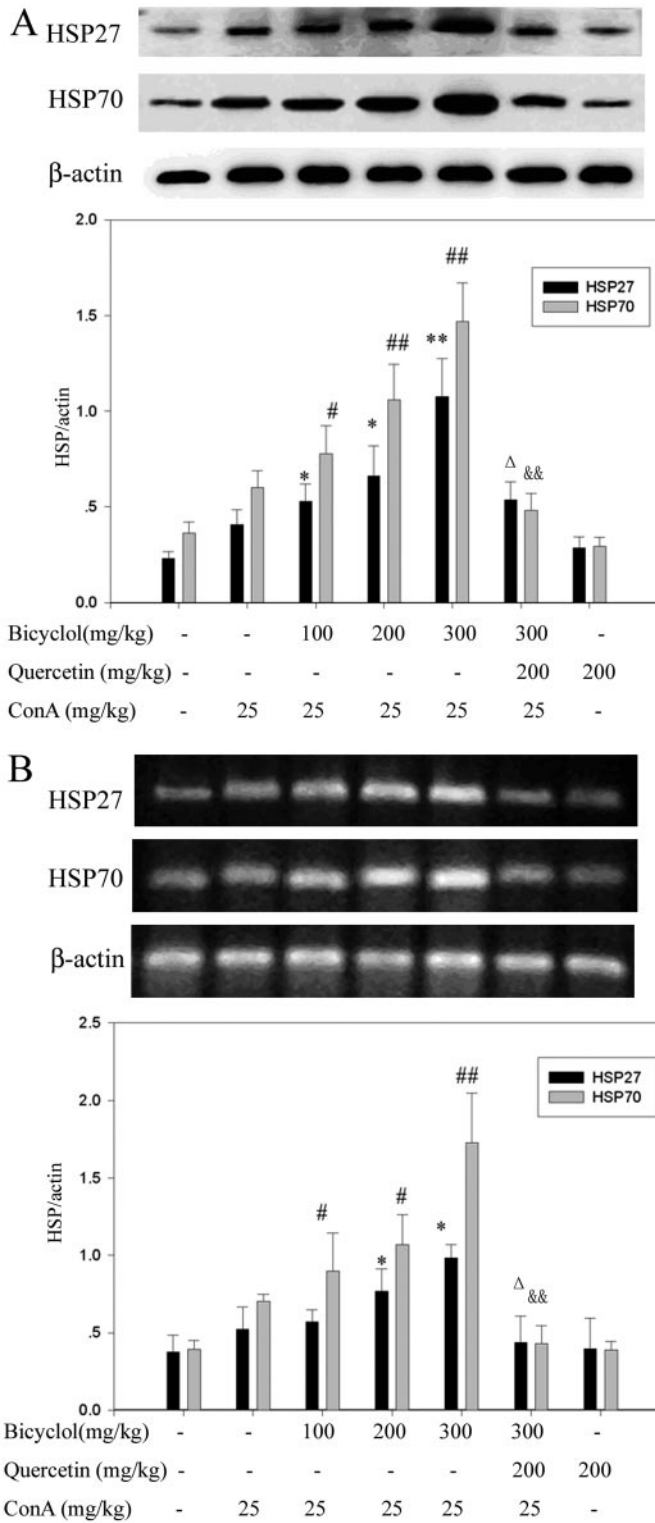


Fig. 2. Dose-effect relationship of bicyclol in inducing hepatic HSP27 and HSP70 of mice injected with ConA. Mice were orally administered bicyclol 100, 200, and 300 mg/kg three times in 24 h. Quercetin 200 mg/kg was administered in association with bicyclol. The mice were injected ConA 1 h after the last administration of bicyclol and were sacrificed 6 h later. A, Western blot of hepatic HSP27 and HSP70. * and #, $P < 0.05$; ** and ##, $P < 0.01$ versus control group; Δ , $P < 0.05$; &&, $P < 0.01$ versus bicyclol 300 mg/kg + ConA group. B, RT-PCR analysis of hepatic HSP27 and HSP70 mRNA. * and #, $P < 0.05$; ** and ##, $P < 0.01$ versus control group; Δ , $P < 0.05$ and &&, $P < 0.01$ versus bicyclol 300 mg/kg + ConA group. A representative from three independent experiments is shown.

There was a good correlation of the accumulation of HSP27 and HSP70 with the expression of HSP27 and HSP70 mRNA, suggesting that the inductive effect of bicyclol on liver HSP27 and HSP70 accumulation is via up-regulation of the transcription of HSP genes. Co-administration of quercetin attenuated the inductive effect of bicyclol on HSP27 (55% decrease, $P < 0.05$) and HSP70 (76% decrease, $P < 0.01$) mRNA expression (Fig. 2B).

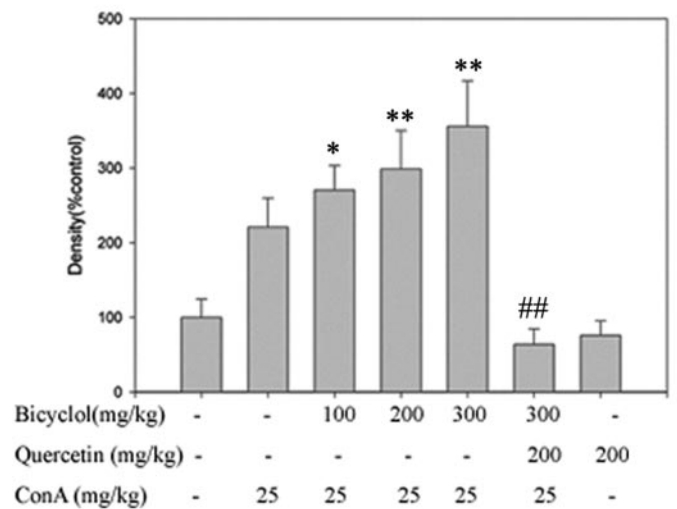
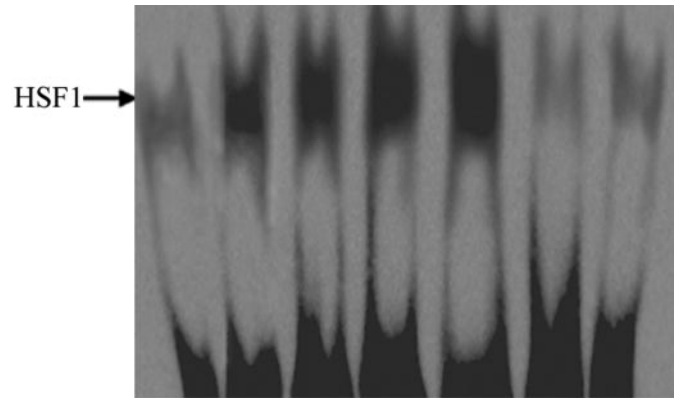


Fig. 3. Effect of bicyclol on the activation of hepatic HSF1 in ConA-treated mice. Mice were orally administered bicyclol 100, 200, and 300 mg/kg three times in 24 h. Quercetin 200 mg/kg was administered associated with bicyclol. The mice were injected ConA 25 mg/kg 1 h after the last administration of bicyclol and were sacrificed 6 h later. HSF1 activity was measured with gel mobility shift assay. *, $P < 0.05$; **, $P < 0.01$ versus control; ##, $P < 0.01$ versus bicyclol 300 mg/kg + ConA. The figure is a representative result from three separate experiments.

TABLE 1

Protection by bicyclol against ConA-induced liver injury was attenuated by quercetin as determined by ALT/AST

Values represent mean \pm S.D., $n = 7$ in each group.

	ALT	AST
	units / liter	
Normal control	37.5 \pm 4.6	38.0 \pm 9.6
ConA	166.4 \pm 53.2*	150.9 \pm 18.6*
Bicyclol + ConA	57.1 \pm 6.1†	66.4 \pm 12.5†
Bicyclol + ConA + quercetin	120.8 \pm 58.8	135.5 \pm 24.7
ConA + quercetin	170.6 \pm 49.8	143.2 \pm 20.4
Quercetin	34.4 \pm 6.5	38.6 \pm 10.1

* $P < 0.01$ vs. normal control.

† $P < 0.01$ vs. ConA group and bicyclol + ConA + quercetin group.

Activation of Heat Shock Factor 1 by Bicyclol in ConA-Treated Mice. Heat shock factor 1 (HSF1) mainly presents in the cytosol of nonproliferating cells, and its HSE binding activity in the nucleus is used as a good indicator of cells being under stressful conditions. As shown in Fig. 3, injection of ConA 25 mg/kg induced HSF1-HSE binding activity (lane 2). Prior administration of bicyclol further elevated ConA-induced activation of HSF1 in a dose-dependent manner (Fig. 3, lanes 3–5). Bicyclol 300 mg/kg was more effective on HSF1 activation than at 100 and 200 mg/kg (100 mg/kg: 1.7-fold increase, $P < 0.05$; 200 mg/kg: 1.98-fold increase, $P < 0.01$; 300 mg/kg: 3.55-fold increase, $P < 0.01$).

This effect was counteracted by coadministration of quercetin (Fig. 3, lane 6). These data suggest that bicyclol may induce HSP27 and HSP70 expression by activating HSF1.

Role of Bicyclol-Induced Hepatic HSP27 and HSP70 in Protecting Against ConA-Induced Mouse Liver Injury. To confirm whether the induction of HSP27 and HSP70 by bicyclol plays a critical role in protecting against ConA-induced liver injury, quercetin, the inhibitor of HSPs, was administered together with bicyclol in mice. Pretreatment of bicyclol 300 mg/kg markedly alleviated ConA-caused liver injury in mice, as indicated by the reduction of serum aminotransferase (Table 1), liver necrosis (Fig. 4A), the release of

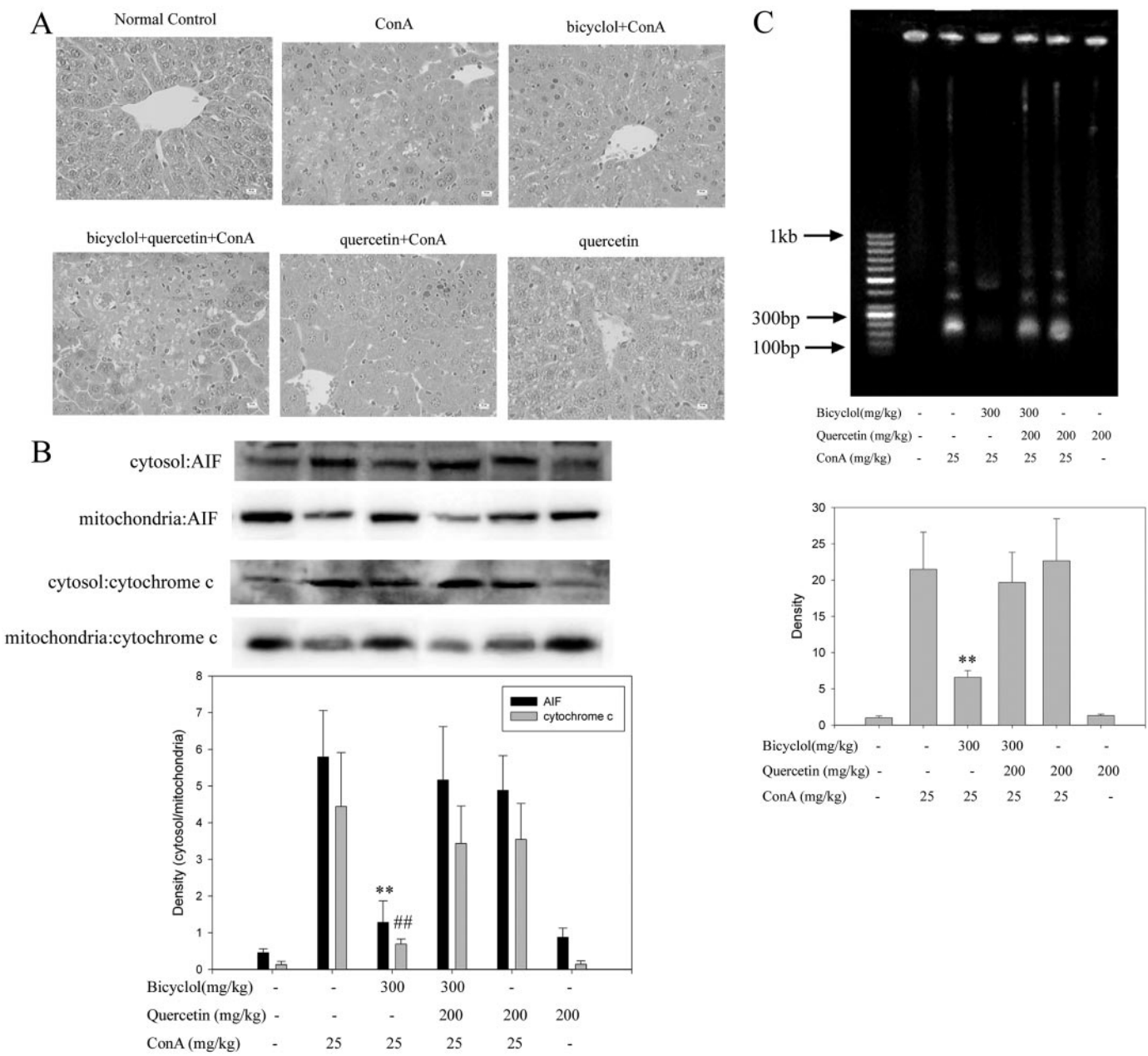


Fig. 4. The role of hepatic HSP27 and HSP70 induced by bicyclol in protection against ConA-induced liver injury in mice. Mice were orally administered bicyclol 300 mg/kg three times in 24 h, and then they were injected with ConA 25 mg/kg 1 h after the last dose of bicyclol. The liver tissues were obtained 6 h after ConA injection for different determinations. A, liver lesions (hematoxylin-eosin stain; scale bar, 50 μ m). B, Western blot of cytochrome c and AIF in mitochondria and cytosol. A representative of three separate experiments is shown. ** and ##, $P < 0.01$ versus ConA, bicyclol + ConA, and ConA + quercetin. C, representative hepatic DNA fragmentation (DNA ladder). **, $P < 0.01$ versus ConA, bicyclol + ConA + quercetin, and ConA + quercetin.

cytochrome *c* (84.4% decrease, $P < 0.01$), and AIF (77.8% decrease, $P < 0.01$) from mitochondria (Fig. 4B) and hepatic DNA fragmentation (69.3% decrease, $P < 0.01$) (Fig. 4C). Preadministration of quercetin 200 mg/kg together with bicyclol 300 mg/kg significantly attenuated all the protective actions of bicyclol, as mentioned above in ConA-challenged mice. However, quercetin itself showed no effect on the above biomarkers in both normal and ConA-treated mice. These results suggest that HSP27 and HSP70 may act as protectors against ConA-induced liver injury in mice.

Liver Injury Induced by ConA Was NF- κ B-Mediated. To further clarify whether the mouse liver injury induced by ConA was mediated by NF- κ B, the proteasome inhibitor MG132, which is a well known NF- κ B inhibitor by blocking degradation of I κ B- α , was used. MG132 reduced serum TNF- α and IL-2 levels 6 h after ConA injection in mouse liver, and bicyclol decreased the levels of TNF- α and IL-2 challenged with ConA (Table 2). These data indicate that the productions of TNF- α and IL-2 induced by ConA were NF- κ B-mediated and that bicyclol decreased TNF- α and IL-2 productions through the direct inhibition of NF- κ B activity. We speculate that bicyclol blocked NF- κ B activation, subsequently inhibited the transcription of TNF- α and IL-2, the target genes of NF- κ B, and finally, mice were protected from ConA-induced hepatic injury.

Bicyclol-Induced HSP27 and HSP70 Inhibits I κ B Degradation and NF- κ B Activation in ConA-Treated Mice. I κ B inhibits the transcriptional activity of NF- κ B by preventing the nuclear translocation of NF- κ B in cytoplasm. Here we show that 1 h after the injection of ConA 25 mg/kg, the degradation of I κ B- α was observed. However, bicyclol alone had no effect on the level of I κ B- α (Fig. 5A). Pretreatment of bicyclol decreased ConA-induced I κ B- α degradation, and this inhibitory effect was in a dose-dependent manner (bicyclol 200 mg/kg: 2.7-fold increase, $P < 0.05$; bicyclol 300 mg/kg: 3.9-fold increase, $P < 0.01$) (Fig. 5, A and B). Co-administration of quercetin counteracted the inhibitory effect of bicyclol on I κ B- α s degradation (69% decrease, $P < 0.01$) (Fig. 5B). Thus, this result suggests that bicyclol may exert its inhibitory effect on the degradation of I κ B- α through its induction of HSP27 and HSP70.

It has become increasingly apparent that the heat shock response can attenuate NF- κ B-mediated activation of inflammatory response. Thus, we further studied the effect of hepatic HSP27 and HSP70 induced by bicyclol on NF- κ B activation in ConA-treated mice. Hepatic NF- κ B was activated after the treatment of ConA in mice, which was inhibited by MG132 (Fig. 5C). Pretreatment of bicyclol inhibited nuclear translocation of NF- κ B p65 subunit and NF- κ B-DNA-binding activity induced by ConA. However, when the hepatic HSP27

and HSP70 synthesis was inhibited with quercetin, the suppression of nuclear translocation of NF- κ B p65 subunit and NF- κ B-DNA binding activity by bicyclol was abrogated, suggesting that HSP27 and HSP70 induced by bicyclol might be involved in the suppression of NF- κ B activation (Fig. 5, D and E).

Interaction of Bicyclol Induced Hepatic HSP27 and HSP70 with IKK and I κ B in ConA-Treated Mice. To further explore the interactions of hepatic HSP27 and HSP70 with IKK and I κ B, a set of coimmunoprecipitations with IKK- α or I κ B- α antibodies was performed using liver tissues isolated from ConA-treated mice. The interactions of HSP27 and HSP70 with IKK- α and I κ B- α were different, because HSP27 efficiently interacted with IKK- α but not with I κ B- α (Fig. 6A), whereas HSP70 interacted with I κ B- α more markedly than with IKK- α (Fig. 6B). Blocking of HSP27 and HSP70 biosynthesis with quercetin reduced these interactions, respectively (Fig. 6, A and B), suggesting that the inhibition of IKK activity and I κ B- α degradation by bicyclol was through its induction of hepatic HSP27 and HSP70, respectively.

Discussion

HSPs were initially identified as cellular response proteins to hyperthermia, and later their induction was also observed after treatment of cells with a number of chemical toxicants. Thus, HSPs have been proposed to play an important role in the protection of cells from injury as "molecular chaperones" (Gething and Sambrook, 1992). Our previous studies showed that bicyclol could protect liver tissues from injury that is caused by chemical toxins, such as ConA (Zhao and Liu, 2001; Li and Liu, 2004). However, it has been unclear whether bicyclol works in protecting liver injury through HSP.

Using a ConA-induced liver injury animal model, we showed that bicyclol may exert such a protective effect by inducing the expression of HSP27 and HSP70, which in turn inhibit NF- κ B-mediated apoptosis. Several lines of evidence support this statement. First, preadministration of mice with bicyclol markedly induced the expression of HSP27 and HSP70 at the levels of mRNA and protein in time- and dose-dependent manners in normal mice and mice challenged with ConA. This increased expression of HSP27 and HSP70 coincided with increased activation of the transcription factor HSF1, which is required for the expression of HSP27 and HSP70. Correlated with these results, preadministration of bicyclol protected against mouse liver injury induced by ConA, as indicated in dramatic decrease of serum ALT/AST levels, liver necrosis, mitochondrial damage, and hepatic DNA fragmentation. Furthermore, quercetin, the inhibitor of HSPs (Jakubowicz-Gil et al., 2002; Masuda et al., 2003), when coadministered with bicyclol, markedly attenuated all of the above protective actions of bicyclol in ConA-treated mice, suggesting that the protective effect of bicyclol on ConA-caused liver injury may be through the induction of HSP27 and HSP70. Together with our previous work showing that induction of HSP27 and HSP70 by bicyclol is well correlated with its inhibitory effect on acetaminophen-induced liver injury (Bao and Liu, 2008), this study further demonstrates that bicyclol also suppresses ConA-induced mouse liver injury by inducing HSP27 and HSP70 in vivo. However, preadministration of quercetin

TABLE 2

Inhibitory effect of MG132 on serum TNF- α and IL-2 challenged with ConA in mice

Values represent mean \pm S.D., $n = 6$ in each group.

Group	TNF- α	IL-2
	pg/ml	
Control	39.19 \pm 5.85	8.63 \pm 4.57
ConA	595.35 \pm 176.39	517.83 \pm 253.93
Bicyclol + ConA	91.56 \pm 24.25*	95.24 \pm 35.78*
MG132 + ConA	76.18 \pm 27.44*	50.71 \pm 27.16*
MG132	61.08 \pm 20.16	8.98 \pm 4.83

* $P < 0.01$ vs. ConA group.

alone three times did not attenuate ConA-induced liver injury. We speculate that there may be a possibility that the concentration of quercetin was not high enough to affect the ConA-induced HSP expression. Because quercetin was previously administered to mice in association with bicyclol three times, the induction of HSPs induced by bicyclol was inhibited. Whereas in the ConA plus quercetin-treated group,

ConA was injected to mice 1 h after the last administration of quercetin, and at that time point, the first two doses of quercetin administered might be mostly metabolized when ConA was injected, so the concentration of quercetin might be too low to inhibit the induction of HSPs by ConA. Thus, quercetin was not shown to affect ConA-induced liver injury.

In addition, our study as presented here suggests a possi-

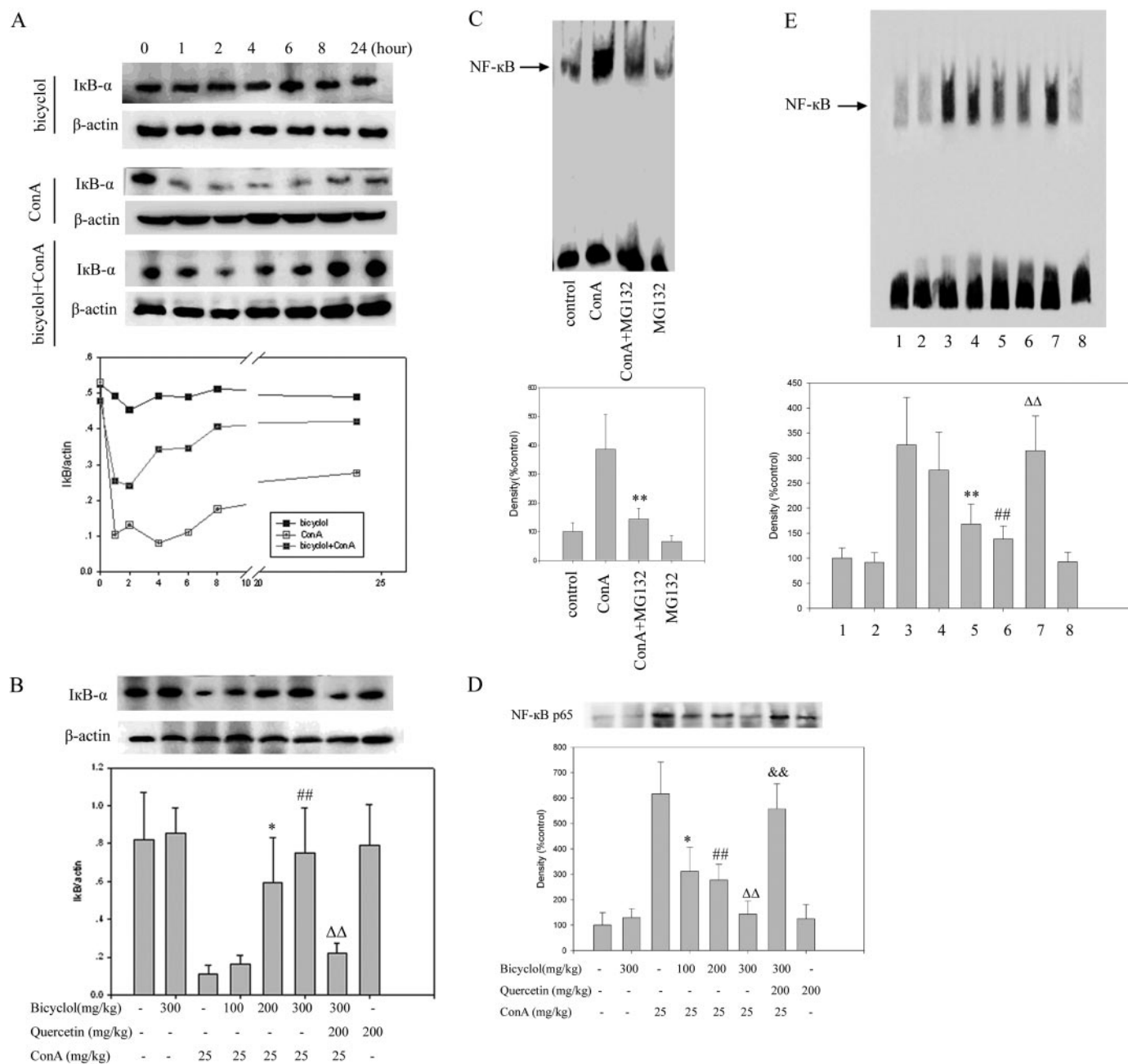


Fig. 5. Inhibition of IκB degradation and NF-κB activation by bicyclol-induced HSP27 and HSP70 in ConA-treated mice. **A**, effect of bicyclol on the degradation of IκB-α. Mice were administered bicyclol 200 mg/kg three times in 24 h, and ConA 25 mg/kg was given 1 h after the last administration of bicyclol. IκB-α was detected at the indicated time. **B**, Dose effect of HSP27 and HSP70 induced by bicyclol on the degradation of IκB-α in ConA-treated mice. Mice were treated as described under *Materials and Methods*. *, $P < 0.05$; **, $P < 0.01$ versus ConA-treated alone; $\Delta\Delta$, $P < 0.01$ versus bicyclol 300 mg/kg + ConA group. **C**, effect of MG132 on NF-κB activation in ConA-treated mice. **, $P < 0.01$ versus ConA-treated alone. **D**, inhibitory effect of bicyclol-induced HSP27 and HSP70 on nuclear localization of NF-κB p65 in ConA-treated mice. *, $P < 0.05$; ## and $\Delta\Delta$, $P < 0.01$ versus ConA-treated alone; &&, $P < 0.01$ versus bicyclol 300 mg/kg + ConA group. **E**, inhibitory effect of bicyclol-induced HSP27 and HSP70 on NF-κB-DNA binding activity in ConA-treated mice. ** and ##, $P < 0.01$ versus ConA-treated alone; $\Delta\Delta$, $P < 0.01$ versus bicyclol 300 mg/kg + ConA group. lane 1, normal control; lane 2, bicyclol 300 mg/kg; lane 3, ConA; lane 4, bicyclol 100 mg/kg + ConA; lane 5, bicyclol 200 mg/kg + ConA; lane 6, bicyclol 300 mg/kg + ConA; lane 7, bicyclol 300 mg/kg + ConA + quercetin; lane 8, quercetin. A representative for each group is shown. Similar results were obtained in three separate experiments.

ble molecular mechanism underlying bicyclol-induced cell survival in ConA-challenged mouse livers (i.e., bicyclol-induced HSP27 and HSP70 may suppress NF- κ B activity and consequently inhibit apoptosis). This assumption is supported by at least three pieces of evidence. First, NF- κ B plays a central role in inducing severe inflammatory diseases such as acute hepatitis (Imose et al., 2004; Ma et al., 2008). In the present study, NF- κ B activation occurred in response to ConA stimulation and TNF- α , and IL-2 productions were inhibited by MG132, an inhibitor of NF- κ B (Fig. 5, D and E). These data indicate that ConA-induced liver injury was mediated by NF- κ B in mice. Second, it has been shown that HSPs can inhibit inflammatory responses and NF- κ B activation (Chen et al., 2004; Chase et al., 2007). Finally, in this study, we found that bicyclol induced the overexpression of HSP27 and HSP70, which in turn suppressed ConA-induced NF- κ B activation in mouse livers. Hence, our study is consistent with previous in vivo and in vitro results (Kammnadiminti and Chadee, 2006; Bhagat et al., 2008), which provide a clue for elucidating the molecular mechanism by which bicyclol protects ConA-induced mouse liver injury by regulating the HSP/NF- κ B pathway.

NF- κ B is a ubiquitous transcription factor that regulates a number of genes involved in inflammation and immune response (Baldwin, 1996), which is normally sequestered in the cytoplasm where it associates with a family of inhibitory proteins known as I κ B. In response to external signals, I κ B is phosphorylated by the IKK complex, and subsequently degraded through a ubiquitin-dependent proteolysis (Yamaoka

et al., 1998). It has been shown that the IKK complex is a potential target for inhibition of NF- κ B pathway by HSPs (Kohn et al., 2002; Broemer et al., 2004). It is noteworthy that we also found that HSP27 and HSP70 induced by bicyclol can increase the cytoplasmic level of I κ B protein in mouse livers after ConA treatment. The result is supported by the observation in which HSPs can inhibit I κ B degradation in response to various stimuli (Uchinami et al., 2002; Chen et al., 2005). In our in vitro study, we found that pretreatment of cycloheximide significantly attenuated the inhibitory effect of bicyclol on I κ B- α degradation in HepG2 cells intoxicated by D-galactosamine, and this attenuation was more potent than that of quercetin (data not shown). This result further indicated that there was a relationship between bicyclol-induced HSPs production and reduced I κ B- α s degradation. Although a study has shown that heat shock can increase I κ B in cytoplasm (Pritts et al., 2000), bicyclol did not seem to trigger the same action to regulate the subcellular localization of I κ B in cytoplasm. This discrepancy may be due to the difference in the nature of heat shock stress and bicyclol treatment.

The inhibitory effects of HSPs on NF- κ B activation are increasingly being demonstrated in different cell systems. Activation of NF- κ B is critically regulated at multiple steps. HSP70 has been found to associate with the p65 subunit of NF- κ B and inhibits the nuclear transport of the latter in T-cells (Guzhova et al., 1997), and HSP27 has been shown to be a ubiquitin-binding protein regulating the degradation of I κ B expression, thereby indirectly influencing NF- κ B activation (Parcellier et al., 2003). Because bicyclol increased HSP27 and HSP70 expression and inhibited ConA-induced NF- κ B activation, their interactions with IKK and I κ B in ConA-treated mouse liver were checked. We observed that HSP27 induced by bicyclol could interact with IKK α , whereas HSP70 interacted with I κ B α . These data suggest that HSP27 and HSP70 physically bind to IKK α and I κ B α , respectively, and thereby may play a cooperative role in stabilizing NF- κ B-I κ B complex and tethering NF- κ B in the cytosol. Similar results were also reported in the case of brain ischemia (Zheng et al., 2008). In particular, one study showed that HSP27 interacts with IKK complex and negatively regulates its activation by TNF- α (Park et al., 2003) in HeLa cells. In contrast, Ran et al. (2004) showed that HSP70 interacts with IKK but not I κ B (Ran et al., 2004). The reasons for these differences are unclear but may stem from the experiment system.

The major focus of the present work concentrated on HSPs in protecting against the acute inflammation that has been shown to contribute to liver injury. Our study not only provides first evidence that HSP27 and HSP70 induced by bicyclol may play critical roles in its protective action against ConA-induced mouse liver injury through suppression of NF- κ B-mediated apoptosis and necrosis in mice but also highlights the potential of bicyclol as a therapy agent against a variety of stress conditions, given that it may protect tissues from injury through the induction of HSPs.

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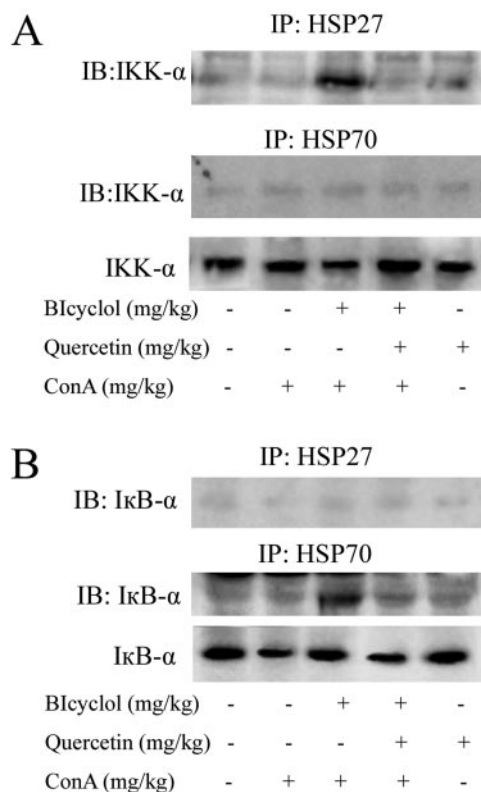


Fig. 6. Interaction of bicyclol-induced hepatic HSP27 and HSP70 with IKK and I κ B in ConA-treated mice. Mice were orally administered bicyclol 300 mg/kg three times in 24 h, and then they were injected with ConA 25 mg/kg 1 h after the last dosing of bicyclol. A, interaction of HSP 27 and HSP70 with IKK- α . B, interaction of HSP27 and HSP70 with I κ B- α . A representative of three experiments is shown.

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