Molecular Pharmacology Fast Forward. Published on February 2, 2009 as DOI: 10.1124/mol.108.053280
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Title page

Induction of heat shock protein 27 and 70 overexpression by bicyclol attenuates concanavalin A -induced liver injury through suppression of NF-κB in mice

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Running title page

Running title: Bicyclol attenuates mouse liver injury

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Number of text pages: 30

Number of tables: 2

Number of figures: 6

Number of references: 37

Number of words in the abstract: 222

Number of words in the introduction: 574

Number of words in the discussion: 1215

Abbreviations: HSPs, heat shock proteins; tumor necrosis factor-α, TNF-α; interleukin, IL; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ConA, concanavalin A; RT-PCR, reverse transcription-polymerase chain reaction; dNTP, deoxy-ribonucleoside triphosphate; TBE, Tris-borate buffer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electropheresis; PVDF, polyvinylidene difluoride; HSF1, heat shock factor-1; AIF, apoptosis inducing factor; EMSA, Electrophoretic mobility shift assay; HSE, heat shock element; mRNA, messenger ribonucleic acid; Co-IP, Co-immunoprecipitation NF-κB, Nuclear factor-kappa B; IκB, inhibitor kappa B; IKK, inhibitor kappa B kinase;

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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 anti-hepatitis drug, has been shown to protect against liver injury in animals. However, it is unclear how bicyclol protects against liver injury. We recently find that bicyclol is an inducer of HSPs. We wondered if 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 in reduction of serum aminotransferases, liver necrosis, the release of cytochrome *c* and apoptosis inducing factor (AIF) from mitochondria and hepatic DNA fragmentation. Correlated with this, bicyclol induced the increase of mRNA and protein levels of hepatic HSP27 and HSP70 in the mice. Correspondingly, the elevated HSP27 and HSP70 suppressed IκB degradation and NF-κB 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 as described above suggest that the anti-hepatitis drug, bicyclol, may protect against liver injury by inducing the expression of hepatic HSP27 and HSP70, and thus consequently inhibit the transcription factor NF-κB-mediated apoptosis and necrosis in liver tissue.

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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 (Kultz, 2005). Thus, an enhanced expression of HSPs in cells 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, geranylgeranylacetone (Nishida et al., 2006) and dibutyryl cyclic adenosine monophosphate (Taakno et al., 1998) can induce the level of HSP70 and increase liver resistance to the damage caused by hepatotoxic compounds or TNF-a. 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 anti-hepatitis drug. This drug has been used for treatment of chronic viral hepatitis B patients in China since 2004. It markedly reduced the elevated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to the normal limits by 50%, and also inhibited hepatitis virus B and C replication by approximately 20% in viral hepatitis B and C patients (Yao et al.,2002). Bicyclol also inhibited replication of

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hepatitis virus in duck viral hepatitis and a HepG2.2.15 cell line (Liu, 2001). Moreover, bicyclol has protective action against experimental 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). Importantly, little noticeable side effect has 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 (data to be published), indicating that bicyclol induces hepatic HSP27 and HSP70 expression through activating transcription of HSPs genes. So it is very interesting to study whether bicyclol may protect liver from chemical-caused injury through HSP-mediated cellular mechanisms.

In an attempt to address these questions, we have 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 find that bicyclol increases the expression of HSP27 and HSP70 in the ConA-treated liver tissues, while the enhanced expression of HSP27 and HSP70 mediate the liver protective roles of bicyclol in the ConA-induced liver injury through inhibition of ConA-stimulated inflammatory responses.

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Materials and Methods

Animals. Male *Kunming* mice weighing 20-22g were supplied by the Animal Center of Chinese Academy of Medical Sciences. Mice were maintained in a 12-hours light/dark cycle at 24°C in a relative humidity of 60% room, 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. Mice were orally administered with 200 mg/kg of bicyclol (dissolved in polyethylene glycol 400) for study of time-course effect on hepatic HSPs. Bicyclol 100, 200 and 300 mg/kg were 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 A.M and 18:00 P.M. on the first day, and 8:00 A.M. on the second day of the experiment. Mice were sacrificed at the indicated time points after the last administration of bicyclol. Quercetin (Sigma, MO, USA) 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.

25 mg/kg of ConA (Sigma, MO, USA) dissolved in pathogen-free saline was injected intravenously to mice 1 hour after the last administration of bicyclol. Control mice were injected with the same volume of saline. Mice were sacrificed 6 hours after ConA injection for assays as described below. In the case of NF-κB inhibition, 2 mg/kg of MG132 [Z-Leu-Leu-Leu-aldehyde (Sigma) dissolved in dimethyl sulfoxide (DMSO)] was injected intraperitoneally (ip) to mice 1 hour before ConA injection.

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Reverse Rranscription-Polymerase Chain Reaction (RT-PCR) assay. Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen CA, USA) following the manufacturer's protocol. RT-PCR was performed using One-Step RT-PCR Kit (Promega, WI, USA). The reaction mixture contains AMV/Tfl reaction buffer 10 μl, dNTP 0.2 mM, 1 μM of each primer, 1mM $MgSO_4$, 0.1u/μl AMV reverse transcriptase and Tfl DNA polymerase, 2μg 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 & Services Company (Shanghai, China): HSP27-forward 5′-CCCACCCTCTATCACGGCTAC-3′ and reverse 5′-GGGCTCAACTCTGGCTATCTC-3′, which leads to a 426-bp product; HSP70-forward 5′-GCGACCTGAACAAGAGCATC-3′ and reverse 5′-GAGCTTGCCCTTGAGACCC-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) and the density of the bands was determined using Gel-Pro Analyzer 4.0 software.

Western Blot Analysis. Mouse liver tissues were homogenized in nondenaturing lysis buffer (Applygen Technologies Inc. Beijing, China). 30μg of sample proteins were

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separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) 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 HSP27, HSP70, inhibitor kappa B-α (IκB-α), NF-κB p65, cytochrome *c*, apoptosis inducing factor (AIF) (Santa Cruz, CA, USA), and inhibitor kappa B kinase-α (IKK-α) (Cell Signaling, MA, USA) in 5% skim milk-TBS-T for 2 hours at room temperature, and then incubated with the horseradish peroxidase-conjugated secondary antibody in skim milk-TBST for 2 hours 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 (EMSA). Liver nuclear extracts for EMSA 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-κB consensus oligonucleotides (5'-GCC TGG GAA AGT CCC CTC AAC T-3') labeled with biotin were synthesized by IDT (NJ, USA). EMSA Kit (Pierce, IL, USA) was used to perform the reaction. The binding reaction (20 μl in total) consists of 10 μg of protein extracts, 20 fmol of biotin labeled DNA, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl 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×TBE buffer（45mM Tris borate, 1mM EDTA), and transferred to a nylon membrane. Then the membrane was detected with the enhanced LAS3000 chemiluminescence system.

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Co-Immunoprecipitation (Co-IP). Mouse livers were lysed in nondenaturing lysis buffer (Applygen Technologies Inc.) The Co-IP assay was performed following the protocol of Co-IP kit (Pierce, IL, USA). Briefly, 50μg of the purified HSP27 or HSP70 antibody was immobilized in 100μl, 50% antibody coupling gel. 300μg protein extracts were incubated with gentle end-over-end mixing for 2 hours at room temperature. Immunoprecipitated complexes were eluted thrice with 50μl elution buffer, boiled and separated by SDS-PAGE, transferred to a PVDF membrane, incubated with IKK-α or IκB-α antibodies, and detected with the enhanced LAS3000 chemiluminescence system.

ALT/AST Determination. The serum level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was determined using commercially kits (Beijing BHKT Clinical Reagent Co., Ltd).

Liver Histopathology. For liver histopathological examination, mouse livers were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The specimen was examined under an NIKON E600 digital camera-equipped microscope, and was analyzed using image Pro-Plus 7200 software.

DNA Fragmentation Assay. Mouse liver DNA was extracted using apoptotic DNA ladder kit (Applygen 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 (ELISA) following the protocol of ELISA kit (eBioscience, CA,USA).

Statistical Analysis. Data were expressed as means ±S.D. Changes in different assays were analyzed by ANOVA followed by the Tukey–Kramer test as the post hoc test.

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$P < 0.05$ 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, 24 hours 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 hours with 1.3-fold increase, $P<0.05$) than the latter (4 hours with 0.8-fold increase, $P<0.05$). Interestingly, pre-administration of bicyclol (200mg/kg for three times within 24 hours) 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 hour ($P<0.01$). The same response was also observed in HSP70, as its level increased by 2.8-fold within 2 hours ($P<0.01$) and remained high until 4 hours, and then gradually decreased to a level equivalent to that for the control at 24 hours (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 (200mg/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,

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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 hours 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 co-administration 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 mg/kg and 300 mg/kg up-regulated hepatic HSP27 (bicyclol 200 mg/kg: about1-fold increase, *P*<0.05; bicyclol 300 mg/kg: about 1.56-fold increase, *P*<0.05) and HSP70 (bicyclol 200 mg/kg: about 1.65-fold increase, *P*<0.05; bicyclol 300 mg/kg: about 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. 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 transcription of HSPs 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).

Activation of Heat Shock Factor 1 (HSF1) by Bicyclol in ConA-Treated Mice. HSF1 mainly presents in the cytosol of non-proliferating cells and its HSE binding activity in nucleus is used as a good indicator of cells being under stressful conditions. As

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shown in Fig. 3, injection of 25 mg/kg ConA 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 lane 3, 4, and 5). 300 mg/kg bicyclol was more effective on HSF1 activation than those were 100 and 200mg/kg bicyclol (100mg/kg: 1.7-fold increase, $P<0.05$; 200mg/kg: 1.98-fold increase, $P<0.01$; 300mg/kg: 3.55-fold increase, $P<0.01$). This effect was counteracted by co-administration of quercetin (Fig.3 lane 6). The 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 critical roles in protecting against ConA-induced liver injury, quercetin, the inhibitor of HSPs, was administered together with bicyclol in mice. Pretreatment of 300 mg/kg bicyclol markedly alleviated ConA-caused liver injury in mice as indicated in reduction of serum aminotransferase (Table 1), liver necrosis (Fig. 4A), the release of cytochrome *c* (84.4% decrease, $P<0.01$) and apoptosis inducing factor (AIF) (77.8% decrease, $P<0.01$)from mitochondria (Fig.4B) and hepatic DNA fragmentation (69.3% decrease, $P<0.01$) (Fig.4C). Pre-administration of 200 mg/kg quercetin together with 300 mg/kg bicyclol 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-κB-mediated. To further clarify whether the

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mouse liver injury induced by ConA was mediated by NF-κB, the proteasome inhibitor MG132, which is a well-known NF-κB inhibitor by blocking degradation of IκB-α, was employed. MG132 reduced serum TNF-α and IL-2 levels 6 hours after ConA injection in mouse liver, and bicyclol decreased the levels of TNF-α and IL-2 challenged with ConA (Table 2). These data indicated that the productions of TNF-α and IL-2 induced by ConA were NF-κB-mediated, and that bicyclol decreased TNF-α and IL-2 productions through the direct inhibition of NF-κB activity. We speculate that bicyclol blocked NF-κB activation, subsequently inhibited the transcription of TNF-α and IL-2, the target genes of NF-κB, and finally mice were protected from ConA-induced hepatic injury.

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

It is becoming increasingly apparent that the heat shock response can attenuate NF-κB-mediated activation of inflammatory response. Thus we further studied the effect of hepatic HSP27 and HSP70 induced by bicyclol on NF-κB activation in ConA treated

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mice. Hepatic NF-κB was activated after the treatment of ConA in mice, which was inhibited by MG132 (Fig.5C). Pretreatment of bicyclol inhibited nuclear translocation of NF-κB p65 subunit and NF-κB-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-κB p65 subunit and NF-κB-DNA-binding activity by bicyclol was abrogated, suggesting that HSP27 and HSP70 induced by bicyclol might be involved in the suppression of NF-κB activation (Figs.5D, 5E).

Interaction of Bicyclol Induced Hepatic HSP27 and HSP70 with IKK and IκB in ConA-Treated Mice. To further explore the interactions of hepatic HSP27 and HSP70 with IKK and IκB, a set of co-immunoprecipitations with IKK-α or IκB-α antibodies were performed using liver tissues isolated from ConA treated mice. The interactions of HSP27 and HSP70 with IKK-α and IκB-α were different, as HSP27 efficiently interacted with IKK-α, but not with IκB-α (Fig. 6A), whereas HSP70 interacted with IκB-α more markedly than with IKK-α (Fig. 6B). Blocking of HSP27 and HSP70 biosynthesis with quercetin reduced these interactions, respectively (Figs. 6A and 6B), suggesting that the inhibition of IKK activity and IκB-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 protection of cells from injury as “molecular chaperones” (Gething and Sambrook, 1992). Our previous

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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 or no.

Using a ConA-induced liver injury animal model, here we show that bicyclol may exert such a protective effect by inducing the expression of HSP27 and HSP70, which in turn inhibit NF-κB-mediated apoptosis. Several lines of evidence support this statement. First, pre-administration 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, pre-administration 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 (Masuda et al., 2003; Jakubowicz-Gil et al., 2002), when co-administration with bicyclol, markedly attenuated all 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 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, pre-administration of quercetin alone for three times did not attenuate ConA-induced liver injury. We speculate that there may be a

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possibility that the concentration of quercetin was not high enough to affect the ConA induced HSPs expression. Because quercetin was prior administered to mice in association with bicyclol for three times, and the induction of HSPs induced by bicyclol was inhibited. Whereas, in the ConA plus quercetin treated group, ConA was injected to mice 1 hour 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 possible molecular mechanism underlying bicyclol-induced cell survival in ConA-challenged mouse livers, i.e. bicyclol-induced HSP27 and HSP70 may suppress NF-κB activity and consequently inhibit apoptosis. This assumption is supported by at least three pieces of evidence. First, NF-κB plays a central role in inducing severe inflammatory diseases like acute hepatitis (Ma et al., 2007; Imose et al., 2004). In the present study, NF-κB activation occurred in response to ConA stimulation and TNF-α as well as IL-2 productions were inhibited by MG132, inhibitor of NF-κB (see Fig.5D, 5E). These data indicated that ConA-induced liver injury was mediated by NF-κB in mice. Secondly, it has been shown that HSPs can inhibit inflammatory responses and NF-κB activation (Chase et al., 2007; Chen et al., 2004). Finally, in this study, we found that bicyclol induced overexpression of HSP27 and HSP70, which in turn suppressed ConA-induced NF-κB activation in mouse livers. Hence, our study consistents with previous *in vivo* and *in vitro* results (Bhagat, et al., 2008; Kammanadiminti and Chadee, 2006) which provide a clue for elucidating the molecular mechanism by which bicyclol protects ConA-induced mouse liver injury by

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regulating the HSPs-NF-κB pathway.

NF-κB 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 IκB. In response to external signals, IκB 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-κB pathway by HSPs(Broemer, et al., 2004; Kohn et al., 2002). Interestingly, we also found that HSP27 and HSP70 induced by bicyclol can increase cytoplasmic level of IκB protein in mouse livers post ConA treatment. The result is supported by the observation in which HSPs can inhibit IκB degradation in response to various stimuli (Chen et al., 2005; Uchinami et al., 2002). In our *in vitro* study, we found that pre-treatment of cycloheximide significantly attenuated the inhibitory effect of bicyclol on IκB-α degradation in HepG2 cells intoxicated by D-galactosamine and this attenuation was more potent than that of quercetin (data to be published). This result further indicated that there was a relationship between bicyclol-induced HSPs production and reduced IκB-α's degradation. Although there is the study to show that heat shock can increase IκB in cytoplasm (Pritts et al., 2000), bicyclol did not appear to trigger a same action to regulate the subcellular localization of IκB in cytoplasm. This discrepancy may be due to the difference in the nature of heat shock stress and bicyclol treatment.

Recently, the inhibitory effects of HSP on NF-κB activation are increasingly being demonstrated in different cell systems. Activation of NF-κB is critically regulated at multiple steps. HSP70 has been found to associate with the p65 subunit of NF-κB and

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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 IκB expression, thereby indirectly influencing NF-κB activation (Parcellier et al., 2003). Since bicyclol increased HSP27 and HSP70 expression and inhibited ConA-induced NF-κB activation, their interactions with IKK and IκB in ConA-treated mouse liver were checked. We observed that HSP27 induced by bicyclol could interact with IKKα, while HSP70 interacted with IκBα. These data suggested that HSP27 and HSP70 physically bind to IKKα and IκBα, respectively, thereby may play a cooperative role in stabilizing NF-κB-IκB complex and tethering NF-κB 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 showed that HSP70 interacts with IKK but not IκB (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-κB 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 induction of HSPs.

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Acknowledgements

We are grateful to Professor Hua Lu at Indiana University School of Medicine for proofreading this manuscript.

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Footnotes

This work was supported by grant from Chinese Ministry of Science and technology (No. 96-901-01-45).

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Figure Legends

Fig.1. Time-course of ConA (A) and bicyclol plus ConA (B) in inducing hepatic HSP27 and HSP70 expression in mice. Bicyclol 200mg/kg was given to mice for three times in 24 hours as described in materials and methods. The mice were intravenously injected a dose of ConA 25mg/kg 1 hour 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. $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$ *vs* 0 hour 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 300mg/kg for three times in 24 hours. Quercetin 200mg/kg was administered in association with bicyclol. The mice were injected ConA 1 hour after the last administration of bicyclol, and sacrificed 6 hours later. (A) Western blot of hepatic HSP27 and HSP70. $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$ *vs* control group, $^{\Delta}P<0.05$, $^{\&\&}P<0.01$ *vs* bicyclol 300mg/kg +ConA group. (B) RT-PCR analysis of hepatic HSP27 and HSP70 mRNA. $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$ *vs* control group, $^{\Delta}P<0.05$, $^{\&\&}P<0.01$ *vs* bicyclol 300mg/kg +ConA group. A representative from three independent experiments is shown.

Fig.3. Effect of bicyclol on activation of hepatic HSF1 in ConA treated mice. Mice were orally administered bicyclol 100, 200 and 300mg/kg for three times in 24 hours. Quercetin 200mg/kg was administered associated with bicyclol. The mice were injected ConA 25mg/kg 1 hour after the last administration of bicyclol, and sacrificed 6 hours

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later. HSF1 activity was measured with gel mobility shift assay. $^{*}P<0.05$,$^{**}P<0.01$ *vs* control, $^{\#\#}P<0.01$ *vs* bicyclol 300mg/kg+ConA. The figure is a representative result from three separate experiments.

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 for three times in 24 hours, and then they were injected with ConA 25mg/kg 1 hour after the last dosing of bicyclol. The liver tissues were obtained 6 hours after ConA injection for different determinations. (A) Liver lesions (hematoxylin-eosin stain, scale bar =50 μm). (B) Western blot of cytochrome *c* and AIF in mitochondria and cytosol. A representative of three separate experiments is shown. $^{**,\#\#}P<0.01$ *vs* ConA, bicyclol+ConA+quercetin and ConA+quercetin. (C) Representative hepatic DNA fragmentation (DNA ladder). $^{**}P<0.01$ *vs* ConA, bicyclol+ConA+quercetin and ConA+quercetin.

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 200mg/kg for three times in 24 hours, and ConA 25mg/kg was given 1 hour 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 in materials and methods. $^{*}P<0.05$, $^{**}P<0.01$ *vs* ConA treated alone, $^{\Delta\Delta}P<0.01$ *vs* bicyclol 300mg/kg +ConA group. (C)Effect of MG132 on NF-κB activation in ConA-treated mice. $^{**}P<0.01$

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vs ConA treated alone. (D) Inhibitory effect of bicyclol-induced HSP27 and HSP70 on nuclear localization of NF-κB in ConA treated mice. $^{*}P<0.05$, $^{\#\#,\Delta\Delta}P<0.01$ *vs* ConA treated alone, $^{\&\&}P<0.01$ *vs* 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. $^{**,\#\#}P<0.01$ *vs* ConA treated alone, $^{\Delta\Delta}P<0.01$ *vs* bicyclol 300 mg/kg + ConA group, Lane 1: Normal control; lane 2: bicyclol 300mg/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.

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

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Table1 Protection by bicyclol against ConA-induced liver injury was attenuated by quercetin as determined by ALT/ AST (U/L).

	ALT	AST
Normal control	37.5±4.6	38.0±9.6
ConA	166.4±53.2**	150.9±18.6$^{\Delta\Delta}$
bicyclol + ConA	57.1±6.1$^{\#\#}$	66.4±12.5$^{\&\&}$
bicyclol + ConA+quercetin	120.8±58.8	135.5±24.7
ConA+quercetin	170.6±49.8	143.2±20.4
quercetin	34.4±6.5	38.6±10.1

Values represent mean ± SD, n=7 in each group.

$^{**,\Delta\Delta}$*P<0.01 vs Normal control.*

$^{\#\#,\&\&}$*P<0.01 vs ConA group and bicyclol+ConA +quercetin group.*

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Table2 Inhibitory effect of MG132 on serum TNF-α and IL-2 challenged with ConA in mice (pg/ml).

Group	TNF-α	IL-2
Control	39.19±5.85	8.63±4.57
ConA	595.35±176.39	517.83±253.93
Bicyclol+ConA	91.56±24.25**	95.24±35.78$^{\#\#}$
MG132+ConA	76.18±27.44**	50.71±27.16$^{\#\#}$
MG132	61.08±20.16	8.98±4.83

Values represent mean ± SD, n=6 in each group.

$^{**,\#\#}$*P<0.01 vs ConA group*

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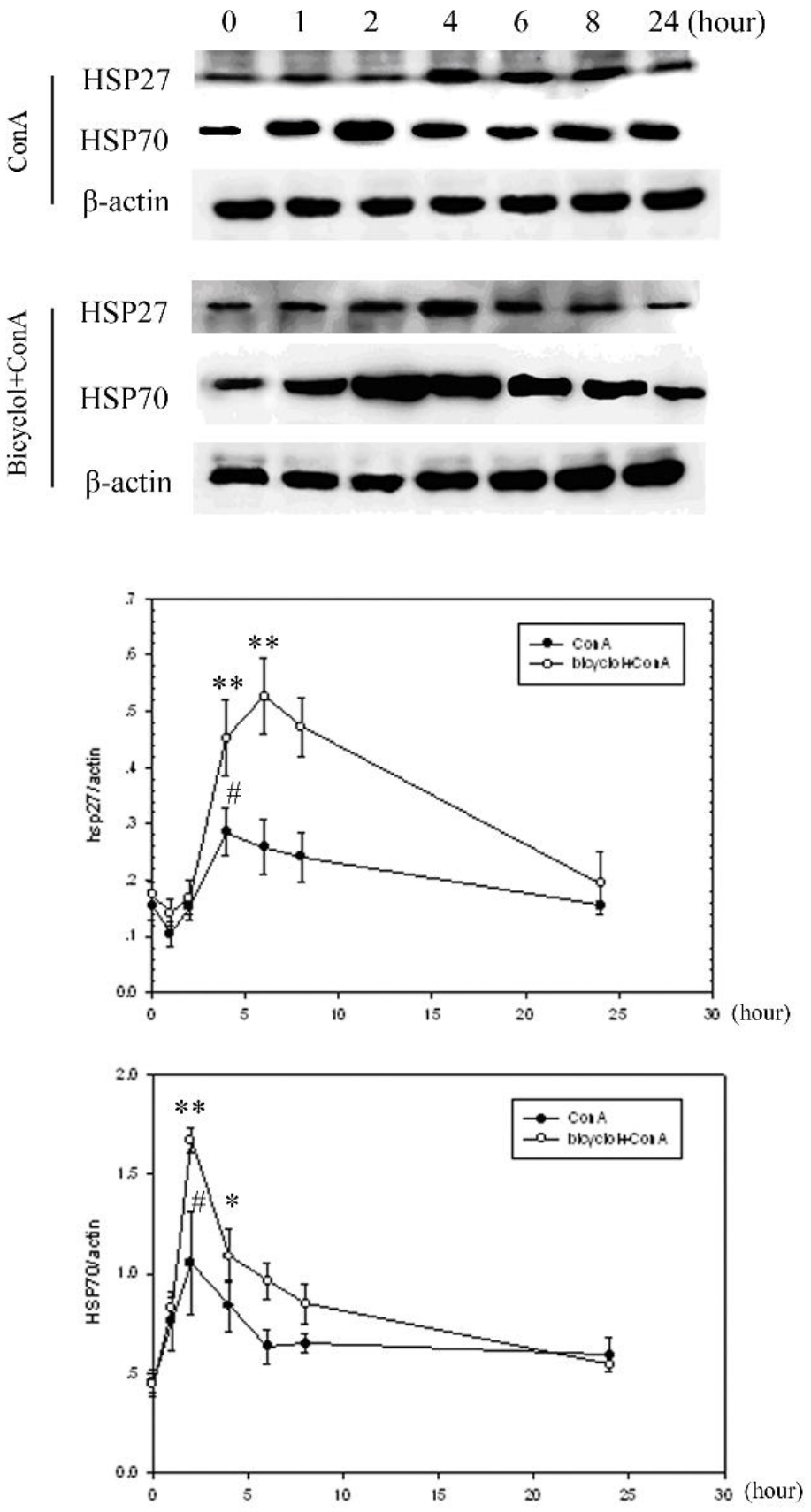
0 1 2 4 6 8 24 (hour)
ConA
HSP27
HSP70
β-actin
Bicyclol+ConA
HSP27
HSP70
β-actin
ConA
bicyclol+ConA
hsp27/actin
**
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#
(hour)
ConA
bicyclol+ConA
HSP70/actin
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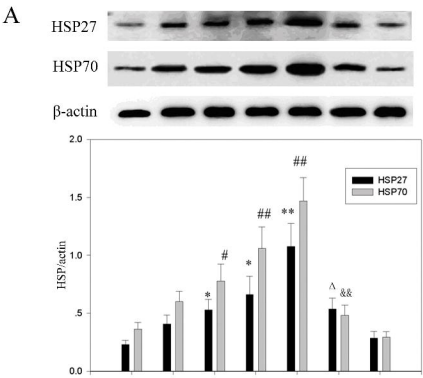
#
*
(hour)

Fig.1


A
HSP27
HSP70
β-actin
HSP/actin
HSP27
HSP70
Bicyclol(mg/kg) - - 100 200 300 300 -
Quercetin (mg/kg) - - - - - 200 200
ConA (mg/kg) - 25 25 25 25 25 -

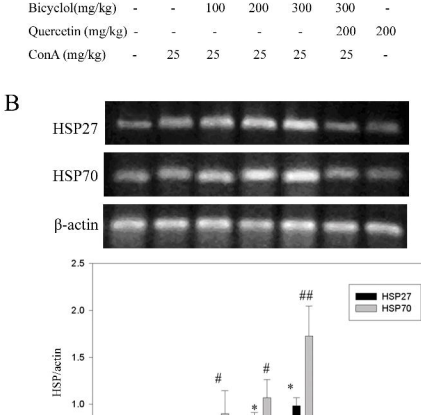

B
HSP27
HSP70
β-actin
HSP/actin
HSP27
HSP70
Bicyclol(mg/kg) - - 100 200 300 300 -
Quercetin (mg/kg) - - - - - 200 200
ConA (mg/kg) - 25 25 25 25 25 -

Fig.2

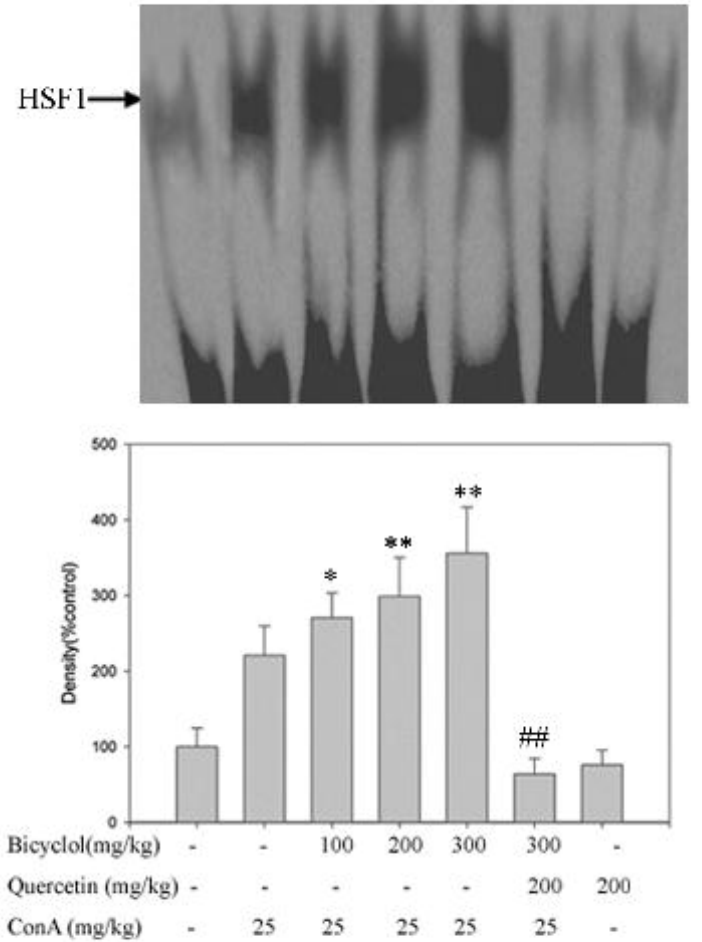
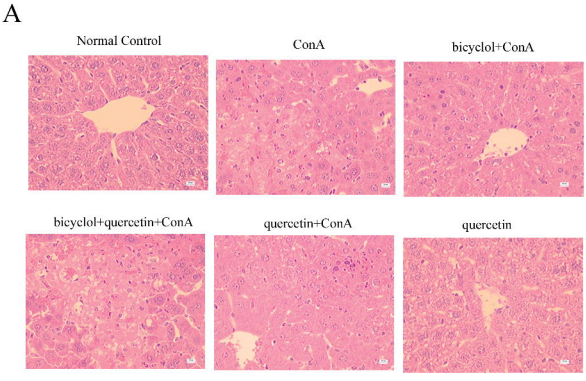
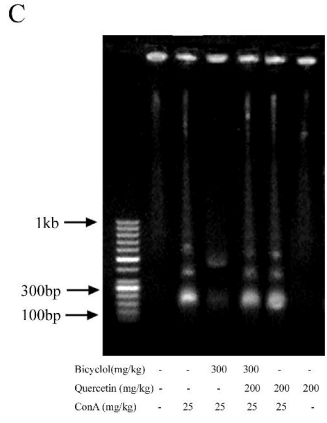

HSF1
Density(%control)
500
400
300
200
100
0
*
**
**
##
Bicyclol(mg/kg) - - 100 200 300 300 -
Quercetin (mg/kg) - - - - - 200 200
ConA (mg/kg) - 25 25 25 25 25 -

Fig.3


A
Normal Control
ConA
bicyclol+ConA
bicyclol+quercetin+ConA
quercetin+ConA
quercetin


C
1kb
300bp
100bp
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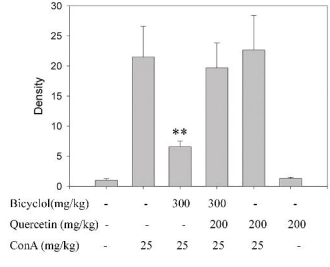
Quercetin (mg/kg) - - - 200 200 200
ConA (mg/kg) - 25 25 25 25 -


Density
30
25
20
15
10
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0
**
Bicyclol(mg/kg) - - 300 300 - -
Quercetin (mg/kg) - - - 200 200 200
ConA (mg/kg) - 25 25 25 25 -

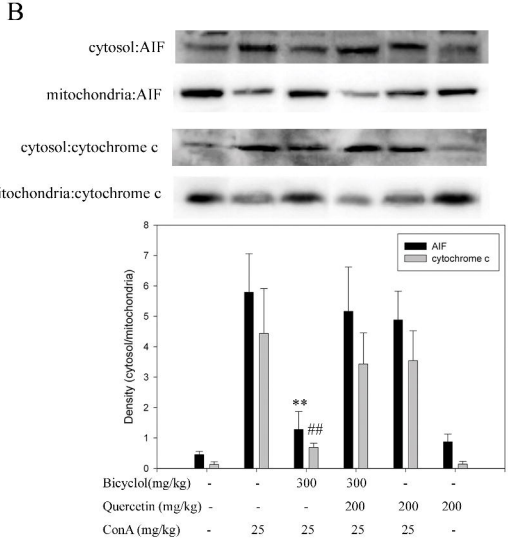

B
cytosol:AIF
mitochondria:AIF
cytosol:cytochrome c
mitochondria:cytochrome c
Density (cytosol/mitochondria)
AIF
cytochrome c
**
##
Bicyclol(mg/kg) - - 300 300 - -
Quercetin (mg/kg) - - - 200 200 200
ConA (mg/kg) - 25 25 25 25 -

Fig.4

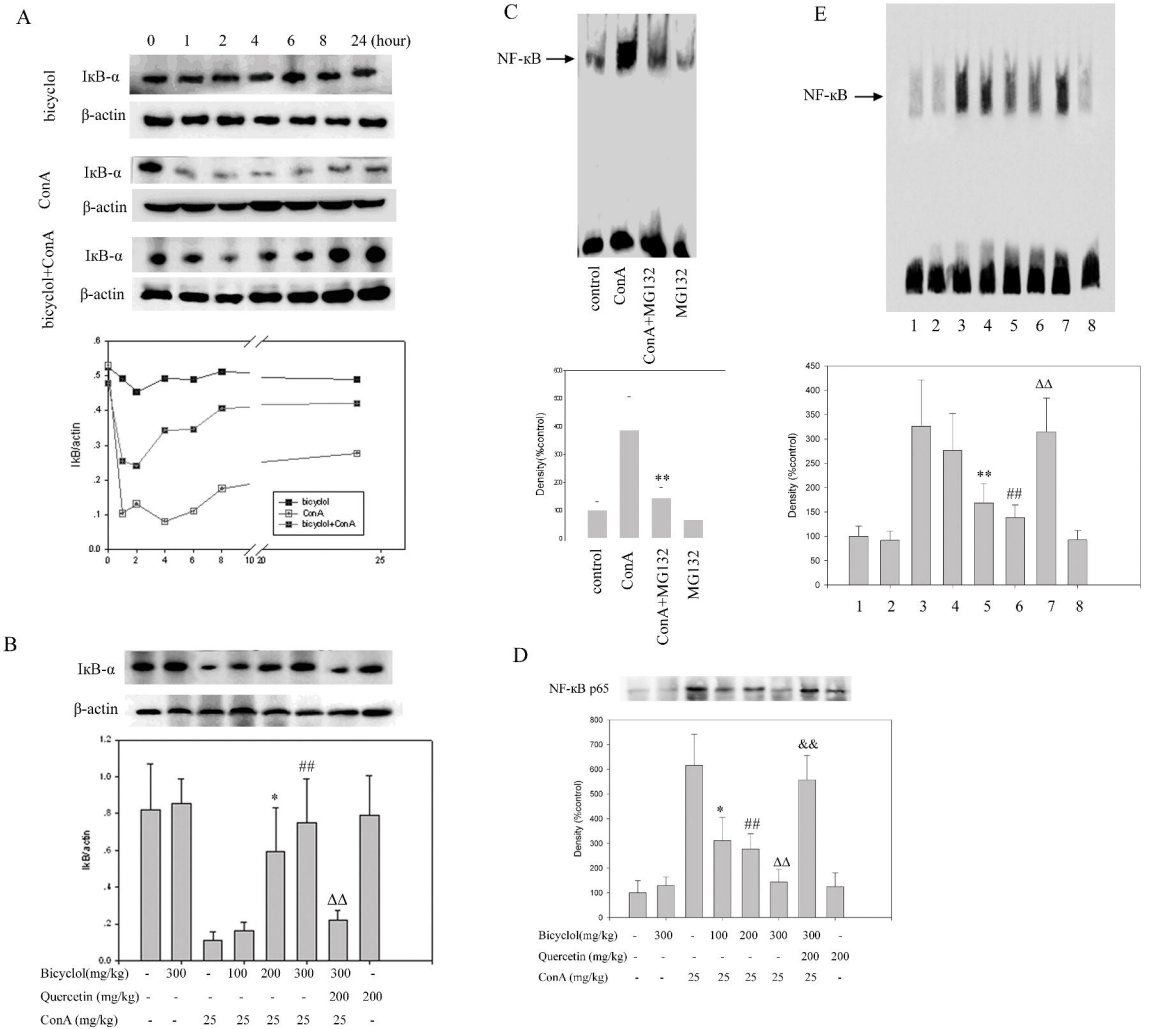
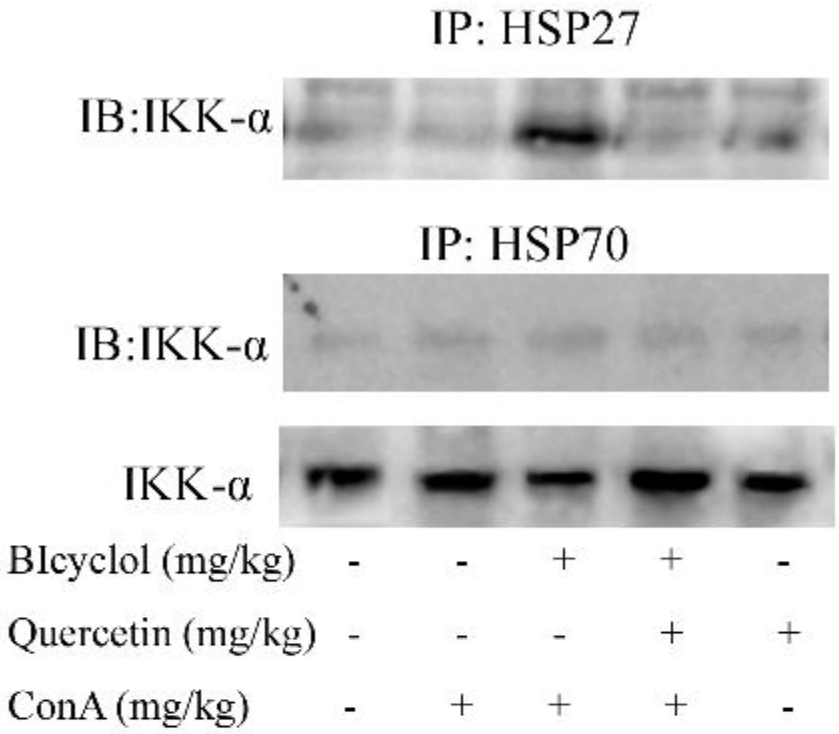

A
0 1 2 4 6 8 24 (hour)
bicyclol
IκB-α
β-actin
ConA
IκB-α
β-actin
bicyclol+ConA
IκB-α
β-actin
IkB/actin
bicyclol
ConA
bicyclol+ConA
B
IκB-α
β-actin
IkB/actin
Bicyclol(mg/kg) - 300 - 100 200 300 300 -
Quercetin (mg/kg) - - - - - - 200 200
ConA (mg/kg) - - 25 25 25 25 25 -
C
NF-κB
control
ConA
ConA+MG132
MG132
Density(%control)
control
ConA
ConA+MG132
MG132
D
NF-κB p65
Density (%control)
Bicyclol(mg/kg) - 300 - 100 200 300 300 -
Quercetin (mg/kg) - - - - - - 200 200
ConA (mg/kg) - - 25 25 25 25 25 -
E
NF-κB
1 2 3 4 5 6 7 8
Density (%control)
1 2 3 4 5 6 7 8

Fig.5

A


IP: HSP27
IB:IKK-α
IP: HSP70
IB:IKK-α
IKK-α
BIcyclol (mg/kg) - - + + -
Quercetin (mg/kg) - - - + +
ConA (mg/kg) - + + + -

B

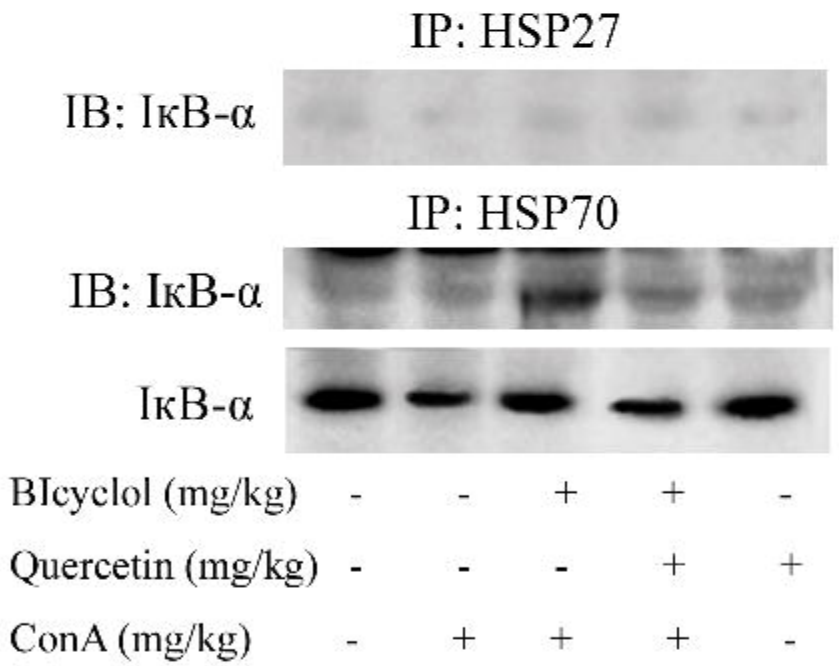

IP: HSP27
IB: IκB-α
IP: HSP70
IB: IκB-α
IκB-α
BIcyclol (mg/kg) - - + + -
Quercetin (mg/kg) - - - + +
ConA (mg/kg) - + + + -

Fig.6