A Sexual Dimorphism Influences Bicyclol-Induced Hepatic Heat Shock Factor 1 Activation and Hepatoprotection

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ABSTRACT

Bicyclol [4,4’-dimethoxy-5,6,5’,6’-bis(methylenedioxy)-2-hydroxy-methyl-2’-methoxycarbonyl biphenyl] is a synthetic hepatoprotectant widely used in clinical practice, but resistance to this treatment is often observed. We found that the hepatoprotective effect of bicyclol was greatly compromised in female and castrated male mice. This study was to dissect the molecular basis behind the sex difference, which might underlie the clinical uncertainty. We compared bicyclol-induced hepatoprotection between male and female mice using acute liver damage models. Inducible knockout by the Cre/loxP system was used to decipher the role of sex hormone receptors in sex-specific hepatoprotection. Western blot, and histopathological analysis were used to assess bicyclol-induced hepatoprotection, as well as practicable solutions to improve the effect of bicyclol.

Introduction

Bicyclol [4,4’-dimethoxy-5,6,5’,6’-bis(methylenedioxy)-2-hydroxy-methyl-2’-methoxycarbonyl biphenyl] is a synthetic hepatoprotectant and has been approved to treat liver injury in China since 2004. Clinical observations have proved that bicyclol treatment significantly decreases serum aminotransferase levels in hepatitis sufferers (Liu, 2009). Bicyclol is also effective in the treatment of acute hepatic injury induced by other causes, such as alcohol, drugs, or ischemia/reperfusion injury (Zhao et al., 2008; Yao et al., 2009). Meanwhile, no notable side effects have been observed during or after bicyclol treatment in the clinical setting.

Although bicyclol seems to be a promising therapeutic option for the treatment of hepatic damage, resistance to bicyclol treatment is frequently observed in clinical practice. It is frequently observed that bicyclol keeps serum aminotransferase at a very low level, although the histologic findings and clinical manifestations indicate ongoing liver damage. Moreover, bicyclol reduces alanine aminotransferase (ALT) levels to a much greater extent than aspartate aminotransferase (AST), resulting in a very high AST/ALT ratio.

Bicyclol-induced hepatic protection has been corroborated in various types of liver damage animal models. For example, acute liver damage induced by carbon tetrachloride (Liu et al., 2005), acetaminophen (Hou et al., 2008), concanavalin A (Bao and Liu, 2009), and D-galactosamine (GalN)/lipopolysaccharide (LPS) can be ameliorated by bicyclol administration. Further studies demonstrated that bicyclol treatment resulted in hepatic activation of heat shock transcription factor 1 (HSF1), which stimulated heat shock protein (HSP) 27 and HSP70 expression, thus leading to the conclusion that the molecular basis of bicyclol-mediated protection against liver injury is the induction of hepatic heat shock proteins (Bao and Liu, 2008, 2009). After reviewing all the available literature on this subject, it is

ABBREVIATIONS:

ALT, alanine aminotransferase; Ana, anastrozole; AST, aspartate aminotransferase; GalN, D-galactosamine; HSP, heat shock protein; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; pIpC, poly deoxyinosinic/deoxyctydilic acid; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione; Ts, testosterone undecanoate.
interesting and surprising to find that all of these studies were conducted in male animals. What happens, then, to the females?

To answer this question, in this study, we subjected both male and female mice to chemical toxicant or drug-induced liver damage, and surprisingly found that bicyclol had few...
effects on females. Mechanically, bicyclol-induced HSF1 activation and HSP induction were compromised in females, which was a result of HSF1 phosphorylation catalyzed by glycogen synthase kinase 3β (GSK3β).

Materials and Methods

Mice. The Cre/loxP recombination system was used to generate HSF1−/− mice that have been described before (Le Masson et al., 2011). Mice containing loxP-flanked HSF1 exons 2–4 (HSF1loxPloxP) were a gift from Dr. Elisabeth S. Christians (University of Utah School of Medicine, Salt Lake City, UT). Mx1 promoter sequence—modified Cre recombinase genes (Mx1-Cre) were from the Jackson Laboratory (Bar Harbor, ME). After a mating of these two strains and a second mating of their progeny, mice that were homozygous for the HSF1 floxed allele and carried the Mx1-Cre transgene were generated (Mx1-Cre+/loxPloxP). Mx1-Cre+/loxPloxP mice were then backcrossed to the Cre+HSF1loxPloxP mice to generate both Mx1-Cre− mice (deletable) and Cre−littermates (nondeletable). To mutate the target gene, 7-week-old mice were administered intraperitoneal injections of 400 μg of poly deoxyinosinic/deoxycytidylic acid (pIpC) every 3 days for a total of three injections. Mx1-Cre−HSF1loxPloxP mice that had received injections of pIpC are hereafter referred to as HSF1loxPloxP mice. Cre+HSF1loxPloxP mice that had received injections of pIpC served as controls, and are referred to as HSF1loxPloxP mice. Genomic DNA was isolated from tail biopsies, and genotyping was performed using polymerase chain reaction. The floxed and wild-type alleles were detected using the following primers: 5′-CTAGTCAGTCCCTAGAGATGACCAG-3′, 5′-AAGCATAGCATCCTGGAAGAGGTAC-3′, and 5′-GTTGTGAGTCAGCTCGTGC-3′, which generated a 482-bp product in the floxed allele, 432-bp product in the wild type, and 324 bp in the knockout allele. Wild-type male and female C57BL/6 mice, 8–12 weeks old (18–26 g), were purchased from Shanghai SLAC Co. Ltd. (Shanghai, China). A total of 456 male mice and 552 female mice were used in this study.

The animals were handled in accordance with standard use protocols, animal welfare regulations, and the institutional guidelines of Shanghai Jiaotong University School of Medicine and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). All the procedures described were approved on June 25th, 2011, by the Animal Use and Care Committee of Shanghai Jiaotong University School of Medicine (approval number: SYXK-2011-0039).

Determination of Lethality. Survival was observed in some groups after GalN/LPS intoxication. The number of survival mice was counted and recorded every half hour from 4 to 12 hours after the GalN/LPS injection, then every 12 hours until the seventh day after intoxication. No deaths were observed after the third day, so mice that survived more than 72 hours were considered to survive indefinitely. If an animal was considered possibly morbid, the condition of the animal was monitored every 15 minutes. The presence of morbid symptoms was determined by an experienced observer with no prior information regarding the treatments and genetic background of the animals. Animals were considered morbid if they were severely immobile, hunched in posture, experiencing severe hypothermia, and/or unresponsive to noise. After signs of morbidity were detected, death was considered unavoidable and the animal was euthanized under anesthesia with isoflurane inhalation. After that, a laparotomy was conducted, and liver failure was confirmed by macroscopic and microscopic examination. Animals that survived to the seventh day were also euthanized under anesthesia, and the successful recovery of hepatic function was confirmed by serum ALT/AST analyses and macroscopic/microscopic examination.

Western Blot Analysis. Harvested liver samples were homogenized and lysed with cell lysis buffer, which contained one protease inhibitor cocktail tablet per 10 ml of Lysis Reagents (Complete; Roche, Indianapolis, IN). Solutions were then clarified by centrifugation (25 minutes at 16,000g). Solubilized proteins were then resolved on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes (WhatmanGE Life Sciences, Little Chalfont, UK). After being blocked with LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE), blots were incubated with anti-Hsp70 (1:500; Cell Signaling Technology, Inc., Danvers, MA), anti-Cleaved Caspase 3 (1:1000; Cell Signaling Technology), anti-GSK3β (1:1000; Abcam, Cambridge, MA), anti-GSK3β pSer9 (1:500; Abcam), anti-GSK3β pThr216 (1:500; Abcam), and anti-β-actin antibodies (1:2000; Santa Cruz Biotechnology, Inc., Dallas, TX). After incubation with IRDye800 secondary antibodies (1:10,000; LI-COR Biosciences), membranes were washed three more times in Tris-buffered saline/0.05% Tween 20. The blot was visualized using an Odyssey infrared imaging system (LI-COR Biosciences). Samples were corrected for background and quantified using Odyssey software. Hsp70, caspase 3, and GSK3β expression levels were normalized to β-actin.

To detect HSF1, nuclear extracts were isolated from harvested liver tissues using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Inc., Waltham, MA) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche). Nuclear protein...
fractions were electrophoresed on 10% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Whatman) by standard procedures. Membranes were blocked with LI-COR blocking buffer. Membranes were then incubated with the same blocking solution containing rabbit polyclonal primary antibodies against HSF1 (1:1000; Cell Signaling Technology, Inc.) and phospho-HSF1 (pSer303, 1:500; Abcam). After washing, membranes were incubated at room temperature for 1 hour in Tris-buffered saline/0.05% Tween 20 buffer with IRDye800 secondary antibodies, and blots were developed as described earlier. All values were normalized to a loading control TATA binding protein (1:2000; Abcam) and expressed as the fold increase relative to control.

**ALT/AST Determination.** Arterial blood was collected by direct puncture of arteriae aorta under anesthesia with isoflurane inhalation. Serum levels of ALT and AST were measured with a standard clinical automatic analyzer (Dimension Xpand; Siemens Dade Behring, Munich, Germany).

**Liver Histopathology.** Hepatic samples were fixed in 10% neutral buffered formalin overnight, dehydrated, embedded in paraffin, sectioned,

![Fig. 3. Bicyclol lost hepatoprotective effect in castrated males. Male mice were subjected to castration, followed by bicyclol (Bic) treatment and GalN/LPS intoxication. (A) Survival of mice after intoxication (n = 20 per group). Bicyclol treatment led to a significant survival advantage only in sham-operated mice by Kaplan-Meier analysis (log-rank test, P < 0.05). (B) Representative immunoblotting results of protein expression of Hsp70 and active caspase 3 in postintoxication livers harvested at 6 (PBS group) or 8 hours (Bic group). (C) Protein bands were quantified and normalized to β-actin. For Hsp70, the mean value obtained from PBS-treated control mice was arbitrarily defined as 1. For caspase 3, the mean value obtained from PBS-treated control mice was arbitrarily defined as 10. There were four mice in each group, and data are expressed as the mean ± S.D. *P < 0.05 versus other groups.](image)

![Fig. 4. The effect of Ts or Ana on bicyclol-induced hepatoprotection in females. Female mice were pretreated with Ts or Ana, followed by bicyclol (Bic) treatment and GalN/LPS intoxication. (A) Survival of mice after intoxication (n = 20 per group). Bicyclol treatment led to a significant survival advantage in Ts- or Ana-pretreated mice by Kaplan-Meier analysis (log-rank test, P < 0.05 versus the single agent–treated groups). (B) Representative immunoblotting results of protein expression of Hsp70 and active caspase 3 in livers harvested at 6 (PBS groups) or 8 hours (Bic groups) after intoxication. (C) Protein bands were quantified and normalized to β-actin. For Hsp70, the mean value obtained from PBS-treated control mice was arbitrarily defined as 1. For caspase 3, the mean value obtained from PBS-treated control mice was arbitrarily defined as 10. There were four mice in each group, and data are expressed as the mean ± S.D. *P < 0.05 versus the single agent groups.](image)
and stained with H&E. For histologic analysis, sections were evaluated in a blinded manner by a pathologist. At least three fields per section were evaluated.

**Drugs and Experimental Design.** Bicyclol (Beijing Union Pharmaceutical Plant, Beijing, China) was suspended in phosphate-buffered saline (PBS) to produce injectable suspensions and was administered by gavage three times (300 mg/kg each time) in 24 hours. One hour after the last administration, the mice were subjected to intraperitoneal injection of GalN (700 mg/kg; Sigma-Aldrich, St. Louis, MO) and LPS (10 µg/kg; from *Escherichia coli* serotype 055:B5; Sigma-Aldrich) to induce acute liver failure. Additional animals in control groups received vehicle (PBS) orally, followed by GalN/LPS injection. In other groups, carbon tetrachloride (0.4% diluted in olive oil; Sigma-Aldrich) was administered intraperitoneally at a single dose of 10 ml/kg at 1 hour after the last administration of bicyclol. Some mice received acetaminophen (dissolved in PBS; Sigma-Aldrich) intraperitoneally at a single dose of 300 mg/kg at 1 hour after the last administration of bicyclol. In separate groups, bicyclol was administered once (300 mg/kg) at 1 hour after the injection of GalN/LPS or acetaminophen. Dosage of bicyclol was used in accordance with previously published work (Wang and Li, 2006; Bao and Liu, 2009). Animals were killed at different time points after intoxication by exsanguination, to obtain blood and liver samples for further analyses. Some male mice were castrated 3 weeks before they were subjected to bicyclol administration and GalN/LPS intoxication. In brief, mice were anesthetized with isoflurane inhalation (0.5–2.0%), and the flow rate of the inhaled agent was adjusted as required to maintain appropriate depth of anesthesia, which continued until the closure of the skin wound. After anesthesia was induced, both testes were exposed and sectioned. Analgesia was induced using bupivacaine (0.5%), a long-acting local analgesic, immediately after surgery and only once. Several drops of bupivacaine were dripped on the suture line before the closure of the skin wound. All of these efforts were made to minimize suffering. The mice in the sham-operation group were subjected to the same procedures, except that no testis was resected.

Some female mice were treated with a single injection of long-term-release testosterone undecanoate (T; 12.5 mg in 0.1 ml per mouse; Xianju Pharmaceutical Co., Ltd., Zhejiang, China) by subcutaneous injection in the neck, and 14 days later, the mice were subjected to bicyclol treatment and GalN/LPS intoxication. Anastrozole (Ana; 25 µg/mouse; Sigma-Aldrich) was administered subcutaneously once daily for 14 consecutive days, and was followed by GalN/LPS treatment. Some female mice were treated with GSK3β inhibitors. Lithium chloride solution (150 mg/kg; Sigma-Aldrich), SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione; dissolved in dimethylsulfoxide; 10 mg/kg; Sigma-Aldrich), or tideglibus (dissolved in dimethylsulfoxide; 10 mg/kg; Cayman Chemical, Ann Arbor, MI) was administered intraperitoneally three times (at the same time point as bicyclol) in 24 hours, followed by GalN/LPS intoxication.

**Statistical Analysis.** All values were reported as the mean ± S.D. Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keul’s test where applicable. Statistical significance was set at *P < 0.05.*

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**Fig. 5.** The capacity of bicyclol (Bic) to induce Hsp70 expression and hepatoprotection was compromised in HSF1−/− (Cre−) mice. (A) Nuclear HSF1 expression in liver tissues was evaluated by western blot analysis, and codetection of TATA binding protein (TBP) was performed to assess equal loading (*n = 4* for each group). HSF1 protein bands were then quantified and normalized to TBP. Data are expressed as the mean ± S.D. The results from Cre-negative control (HSF1+/+) mice were arbitrarily defined as 10. *P < 0.05 versus Cre-negative controls. (B) Survival of mice after intoxication (*n = 10* for each group). Bicyclol treatment did not lead to significant survival advantages in HSF1−/− mice by Kaplan-Meier analysis (log-rank test, *P < 0.05* between Bic-treated HSF1−/− and HSF1+/+ mice). (C) Representative immunoblotting results of protein expression of Hsp70 and active caspase 3 in livers harvested at 8 hours after intoxication. (D) Protein bands were quantified and normalized to β-actin. For Hsp70, the mean value obtained from HSF1+/+ mice was arbitrarily defined as 10. For caspase 3, the mean value obtained from HSF1+/+ mice was arbitrarily defined as 1. There were four mice in each group, and data are expressed as the mean ± S.D. *P < 0.05 versus HSF1+/+ mice. (E) Representative H&E-stained sections from postintoxication livers harvested at 6 (PBS groups) or 8 hours (Bic groups) (original magnification 200×).
Results

There Was a Great Sex Difference in Bicyclol-Induced Hepatoprotection. In the study of bicyclol-induced hepatoprotection, we used a previously described protocol (Wang and Li, 2006). Three doses of bicyclol (300 mg/kg) were administered orally to mice within 24 hours. One hour after the last dose of bicyclol, the mice were subjected to intraperitoneal injection of GalN (700 mg/kg/LPS (10 μg/kg)). Without bicyclol treatment, deaths of mice (male or female) occurred at 5.5 hours, and all mice died within 2 hours thereafter. Bicyclol treatment led to a survival rate of over 90% in male mice, but showed few protective effects in female mice (Fig. 1A). Blood aminotransferases (ALT and AST) increased in female mice despite treatment with bicyclol, and all of them died within 10 hours, thereby precluding further time-course studies (Fig. 1, C and D). It is worth noting that the elevation of serum ALT was not very significant and was not comparable with AST, even at 9 hours after the intoxication, and the mice were going to die within minutes. The macroscopic and microscopic pathologic findings were consistent and are shown in Fig. 1, B and E. The sex difference remained when bicyclol was given after GalN/LPS intoxication (Supplemental Fig. 1), or in other liver injury models such as acetaminophen (Supplemental Fig. 2) and carbon tetrachloride (Supplemental Fig. 3). These findings indicate that only males are protected against acute liver damage by bicyclol, which leads to a high AST/ALT ratio and no protection in females.

Bicyclol Induced Hsp70 Expression in Male, but Not Female, Livers. Previous studies (Bao and Liu, 2008, 2009) have demonstrated that bicyclol induces hepatic HSPs, which constitute the molecular basis of the hepatoprotection afforded by bicyclol. So we sought to determine the role of a key heat shock protein, Hsp70, in the sex difference of bicyclol-induced hepatoprotection. At different time points after GalN/LPS intoxication, livers were harvested and total protein was subjected to immunoblotting analysis. A representative result was shown in Fig. 2. In line with the serum and histologic findings, bicyclol induced Hsp70 expression in male livers but failed to do so in females. Cleaved caspase 3, a key mediator of mammalian cell apoptosis and an indicator of liver injury in this setting, was negatively related to the expression of Hsp70.

Bicyclol Lost Hepatoprotective Effect in Castrated Male Mice, but Protected Female Mice when Administered Together with Exogenous Testosterone or Anastrozole. To further determine if sex hormones were behind the sexual dimorphism in bicyclol-induced hepatic protection,
we removed testicles of male mice, and subjected them to GalN/LPS intoxication 3 weeks later. Although sham-operated mice were protected as expected, bicyclol failed to induce hepatic Hsp70 and protect the castrated males (Figs. 1E and 3). We next administered Ts to female mice, and subjected the mice to GalN/LPS intoxication 2 weeks later. We also explored the effect of Ana, an aromatase inhibitor that prevented estrogen synthesis from androgens. Ts or Ana alone did not induce hepatic Hsp70 expression and hepatoprotection, but when administered with bicyclol, the drug combination showed outstanding synergistic effects and protected females (Figs. 1E and 4).

**HSF1 Was Necessary to the Hepatoprotective Effect of Bicyclol in Males and Testosterone-Treated Females.** To figure out what was behind the sexual dimorphism of bicyclol-induced Hsp70 expression and hepatoprotection, we first used the HSF1−/− mice to explore the role of HSF1 in this setting. Hsp70 is one of the inducible heat shock proteins that play essential roles in protecting cells against stress (Hu et al., 2007), and mammalian HSF1, a protein well known as the major transcriptional regulator of the heat shock response, is required for inducible HSP expression (McMillan et al., 1998). Systematic knockout of HSF1 leads to multiple cellular and developmental defects, decreased body weight, and postnatal growth retardation (Xiao et al., 1999). So we used the Cre/loxP recombination system and an interferon-responsive Mx1-Cre transgene, a method of gene targeting that allowed the inducible inactivation of a target gene in adult mice (Kuhn et al., 1995). Both Mx1-Cre−HSF1loxP/loxP (HSF1−/−) and Mx1-Cre−HSF1loxP/loxP littermates (HSF1+/−) were subjected to injections of the interferon inducer plpC, and the successful disruption of the target gene was confirmed by immunoblotting analysis (Fig. 5A). Although bicyclol showed remarkable protection in HSF1+/− control mice, it failed to induce Hsp70 expression and protect against liver failure in HSF1−/− mice (Fig. 5, B–D). These results indicate that the effect of bicyclol is based on HSF1, and suggest that the variation in HSF1 activity after bicyclol treatment may be responsible for the practice variation and clinical uncertainty in the outcome of the pharmacologic treatment.

**Bicyclol Induced a Suppressive Phosphorylation (Serine 303) of HSF1 in Female Livers, Which Could Be Diminished by Simultaneous Administration of Testosterone or GSK3β Inhibitors.** Phosphorylation of HSF1 on serine 303 by GSK3β was reported to mediate the negative control exerted on the activation domains of HSF1 and inhibit transcriptional activation of heat shock genes (Chu et al., 1996). To explore the possible role of the phosphorylated serine 303 on bicyclol-induced transcriptional activation of HSF1, both male and female mice were given three doses of bicyclol, and 1 hour after the last dose, the livers were harvested and subjected to immunoblotting analysis. As shown in Fig. 6A, bicyclol induced equal amounts of nuclear HSF1 accumulation in both sexes. However, serine 303 was markedly phosphorylated only in female livers, which could be attenuated by simultaneous administration of testosterone (Fig. 6B). SB216763 and tidegulubis (both are GSK3β inhibitors, Fig. 6C) also significantly decreased HSF1 phosphorylation, indicating that serine 303 phosphorylation and the repression of HSF1 activity in females were a result of GSK3β catalytic activity.

**Testosterone Was Necessary for Bicyclol to Inhibit Hepatic GSK3β Activity.** The aforementioned results implied that bicyclol did induce nuclear HSF1 accumulation in female livers, but failed to induce full activation of heat shock response because of GSK3β-catalyzed HSF1 phosphorylation. To compare hepatic GSK3β activity between males and females, liver samples were subjected to immunoblotting analysis using two phosphospecific GSK3β antibodies to probe phosphorylated serine 9 and activity-enhanced phosphorylated tyrosine 216, respectively. As shown in Fig. 7, GSK3β was constitutively active in livers of both sexes. Bicyclol treatment inhibited GSK3β activity in male livers. However, inhibition of GSK3β in female livers could be observed only when administered with bicyclol/testosterone.

**Simultaneous Administration of GSK3β Inhibitors and Bicyclol Protected Female Mice against GalN/LPS-Induced Liver Failure.** The aforementioned results suggested the sexual dimorphism in bicyclol-induced hepatoprotection was based on bicyclol-induced sexual dimorphism in hepatic GSK3β activity. If that was true, GSK3β inhibitors and bicyclol should have a synergistic effect in females. So we used three different GSK3β inhibitors, lithium chloride solution, SB216763, and tidegulubis, which were administered with bicyclol to test the hypothesis. Although these drugs themselves had few effects on GalN/LPS-induced liver damage, any one of the three produced a remarkable synergistic effect with bicyclol in the induction of hepatic Hsp70 expression and hepatoprotection in females (Fig. 8).
Discussion

Protecting the liver from various types of injury remains a major challenge of pharmacotherapy, considering that impairment of the liver is a worldwide problem resulting from a lot of etiologies. The fact that the number of hepatoprotective drugs successfully used in clinical practice is actually very limited accents the clinical importance of bicyclol, a synthetic hepatoprotectant which has been demonstrated to protect against hepatotoxicant-induced, immune-related, or ischemia-mediated liver injury. Moreover, in clinical trials and application, bicyclol shows a certain level of activity to inhibit hepatitis virus replication (Liu, 2009). Studies also indicate that bicyclol has the chemopreventive potential for liver carcinogenesis induced by carcinogens (Zhu et al., 2006; Sun et al., 2012). The reported experimental and clinical results indicate that more extensive comparative experimental studies with bicyclol should be conducted to dissect its molecular basis, and if possible, to improve its clinical effects.

Fig. 8. The effect of GSK3β inhibitors (LiCl solution, SB216763, and tideglusib) on bicyclol-induced hepatoprotection in females. Female mice were treated with one of the three GSK3β inhibitors plus bicyclol (or PBS), followed by GalN/LPS intoxication. (A) Survival of females after intoxication (n = 10 per group). Significant survival advantages were observed in groups treated with GSK3β inhibitors plus bicyclol (Bic) as compared with Bic/dimethylsulfoxide (DMSO)-treated mice (log-rank test, P < 0.05). GSK3β inhibitors themselves did not show survival advantages as compared with PBS/DMSO-treated mice (P > 0.05). (B) Representative immunoblotting results of protein expression of Hsp70 and active caspase 3 in livers harvested at 6 (PBS group) or 8 hours (Bic group) after intoxication. (C) Protein bands were quantified and normalized to β-actin. There were four mice in each group, and data are expressed as the mean ± S.D. *P < 0.05 versus the single agent groups. (D) Representative H&E-stained sections from postintoxication livers harvested at 6 (PBS groups) or 8 hours (Bic groups) (original magnification 200×).
In the current study, we reconfirmed the outstanding protective effect afforded by bicyclol in males. But in female and castrated male mice, bicyclol had few effects on ongoing liver damage, although the serum ALT level was greatly reduced. This was true in all types of liver damage models we tested (Fig. 1; Supplemental Figs. 1–3). Such contradictory outcomes can be easily and frequently observed in the clinical setting. Our finding is very important, not only because a lot of females suffer from hepatic damage and are in need of efficient treatment, but also because hypogonadal testosterone levels are common in males, which may invalidate bicyclol treatment in male patients, too. It has been reported that low testosterone levels in men occur with increasing age, and are frequently observed in the primary care setting, particularly in patients with obesity, metabolic syndrome, and acute inflammation (Schneider et al., 2009). All of these conditions can be related to liver injury, which leads to the application of hepatoprotectants. Liver diseases and cancer are associated with very low testosterone levels (Grossmann et al., 2012). For example, Child-Pugh grades B and C hepatic insufficiency in men results in a significant reduction of both total and free testosterone levels (Zifroni et al., 1991). Alcohol administration also results in acute suppression of testosterone in men (Vatsalya et al., 2012), and patients with alcoholic cirrhosis show hypogonadism and feminization associated with sex hormone imbalance due to enhanced aromatization of testosterone (Pignata et al., 1997). Low serum testosterone is also a special feature of hepatocellular carcinoma (Lampropoulou-Karatzas et al., 1993). Since so many users of bicyclol are possibly hypogonadal, it is not difficult to understand why resistance to bicyclol treatment is frequently observed in clinical practice.

One of the major findings of this study is that bicyclol induces sex-specific hepatoprotection based on a sex-specific HSF1/Hsp70 response, in which androgen matters a lot. It is interesting because sex-biased activation of hepatic HSF1 by bicyclol may underlie the confusing observations in clinical practice. Sex-biased HSF1 activation and heat shock response have been reported before. For example, nuclear HSF1 accumulation and synthesis of Hsp70 were only observed in neurons of testosterone-treated rats, but not in estradiol-treated rats (Papasozomenos and Shanavas, 2002). Synthesis of Hsp70 is regulated by HSF1, which is the master regulator of the heat shock response mediating the inducible rapid, massive, transient, and almost exclusive transcription of heat shock protein genes (Rabindran et al., 1991). Previous reports (Bao and Liu, 2008, 2009) have suggested that HSF1 was the key molecular mediator of the pharmacologic effects of bicyclol. In the current study, by using HSF1 knockout mice, we were able to substantiate the point. HSF1 activation is a multistep and tightly regulated process. In mammalian cells, heat shock induces a transition of the HSF1 protein from monomer to trimer, which is the key step toward nuclear translocation and DNA binding of HSF1. After trimerization, the transcriptional activity of HSF1 is correlated with increased phosphorylation at a number of serine residues (Rabindran et al., 1993). For example, phosphorylation of serine 230 promotes inducible transcriptional activity of HSF1 (Holmberg et al., 2001). On the contrary, sequential phosphorylation of serine 307 and 303 represses the activity of HSF1 (Chu et al., 1996). In the current study, we found that bicyclol-induced nuclear HSF1 accumulation was comparable between males and females, suggesting that HSF1 trimerization and nuclear translocation might not be responsible for its sexual dimorphism. We then evaluated the phosphorylation of two serine residues of HSF1 (pSer230 and pSer303) and found that there was no difference in hepatic pHSF1 (Ser230) between males and females after bicyclol treatment (data not shown). However, the results from pSer303 detection showed a significant difference between males and females. Subsequent studies revealed that hepatic activity of GSK3β, the kinase responsible for phosphorylation of Ser303 in HSF1 (Chu et al., 1996), was influenced by bicyclol. As shown in Fig. 7, GSK3β was constitutively active in both male and female livers, but its activity was inhibited by bicyclol treatment in males or testosterone-treated females, which was consistent with the observation of reduced pHSF1 (S303) expressions in these mice (Fig. 6). The schematic outline of how bicyclol and testosterone influenced the heat shock response in the liver is presented in Fig. 9.

It was previously reported (Papasozomenos and Shanavas, 2002) that testosterone prevented the heat shock–induced overactivation of GSK3β in nerve tissues, which was consistent with our findings in hepatic GSK3β activities. Although further study is clearly needed to clarify how testosterone is involved in GSK3β inactivation, these experiments indicate that bicyclol induces hepatic HSF1 activation because 1) it induces nuclear HSF1 accumulation, an effect independent on testosterone, and 2) it inhibits GSK3β activity to promote HSF1 transcriptional activity, an effect dependent on testosterone. Our observations are consistent with previous reports indicating that GSK3β negatively regulates both DNA-binding and transcriptional activities of HSF1 (Xavier et al., 2000) and inhibition of GSK3β ameliorates GalN/LPS-induced liver injury in males (Chen et al., 2012). The dosage of GSK3β inhibitors we used had few effects in the prevention of acute liver failure in females, but led to remarkable Hsp70 induction and hepatoprotection when administered with bicyclol. These
observations further confirmed our conclusion that GSK3β was implicated in the regulation of hepatic HSF1 activity and pharmacological action of bicyclol.

Our results demonstrate that the poor effects of bicyclol in female or castrated male mice can be markedly improved by the simultaneous administration of testosterone, aromatase inhibitors, or GSK3β inhibitors, which produce a synergistic and remarkable promoting effect with bicyclol to activate HSF1. These findings are of clinical importance because testosterone, aromatase inhibitors, or GSK3β inhibitors are now in clinical use, and the protocol for drug combination can easily be applied to the clinical setting, although further studies, including monitoring of side effects of the new multidrug protocol, are clearly required. Our findings about the enhanced activation of HSF1 by testosterone and the underlying mechanisms are worth further study because HSF1 and HSPs constitute the molecular basis of many other drugs. Further studies targeting the relationship between testosterone and GSK3β might help to develop new hepatic protectants which are not sex-biased.

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Authorship Contributions
Participated in research design: M. Zhang, Jianjun Zhang. Conducted experiments: Chen, Jianjun Zhang, Han, Dai. Performed data analysis: Kong, Xu, Xia. Wrote or contributed to the writing of the manuscript: M. Zhang, Jianjun Zhang.

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