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

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ABBREVIATIONS: ALT, alanine aminotransferase; Ana, Anastrozole; AST, aspartate aminotransferase; Bic, bicyclol; DMSO, dimethyl sulphoxide; GalN,
D-galactosamine; GSK3β, glycogen synthase kinase 3β; HE, hematoxylin and eosin; HSF1, heat shock transcription factor 1; Hsp70, heat shock protein 70; LiCl, Lithium chloride solution; LPS, lipopolysaccharide; PBS, phosphate buffered saline; pIpC, poly deoxyinosinic/deoxyctydyllic acid; Ts, testosterone undecanoate.
ABSTRACT

Bicyclol is a synthetic hepatoprotectant widely used in the clinical practice, but resistance to its treatment is often observed. We found that the hepatoprotective effect of bicyclol was greatly compromised in female or 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 by using acute liver damage models. Inducible knockout by Cre-loxp system was employed to decipher the role of heat shock transcription factor 1 (HSF1). Functional experiments, western blot and histopathological analysis were used to determine the key causative factors which might antagonize bicyclol in female livers. HSF1 activation and heat shock protein (Hsp70) expression, which were responsible for bicyclol-induced hepatoprotection, were compromised in female and castrated male livers. Compromised HSF1 activation was a result of HSF1 phosphorylation at serine 303, which was catalyzed by glycogen synthase kinase 3β (GSK3β). Testosterone was necessary for bicyclol to inhibit hepatic GSK3β activity. Administration of testosterone or GSK3β inhibitors restored bicyclol-induced protection in females. Bicyclol induces sex-specific hepatoprotection based on a sex-specific HSF1/Hsp70 response, in which testosterone and GSK3β play key roles. Because a lot of patients suffering from liver diseases are associated with very low testosterone levels, our results give a possible explanation for the clinical variation in bicyclol-induced hepatoprotection, as well as practicable solutions to it.
Introduction

Bicyclol is a synthetic hepatoprotectant and has been approved to treat liver injury in China since 2004. Its chemical name is 4,4’-dimethoxy-5,6,5’,6’-bis(methylenedioxy)-2-hydroxy-methyl-2’-methoxycarbonyl biphenyl. 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, for example, alcohol, drugs or ischemia/reperfusion injury (Yao et al., 2009; Zhao et al., 2008). Meanwhile, no noticeable 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 the clinical practice. It is frequently observed that bicyclol keeps the serum aminotransferase at a very low level, though the histological 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, CCl₄ (Liu et al., 2005), acetaminophen (Hou et al., 2008), concanavalin A (Bao and Liu, 2009) and D-galactosamine (GalN)/lipopolysaccharide (LPS) induced acute liver damage 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 HSP27 and HSP70 expression (Bao and Liu, 2008; Bao and Liu, 2009). Moreover, the protective effects of bicyclol on mouse liver injury can be markedly compromised by quercitin, an inhibitor of heat shock protein biosynthesis, 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; Bao and Liu, 2009). After reviewing all the available literature on this subject, it is interesting and surprising to find that all these studies were conducted in male animals. Then, what happens 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 was 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 to 4 (HSF1\(^{loxp/loxp}\)) were a kind gift from Dr. Elisabeth S. Christians (University of Utah School of Medicine, Salt Lake City, USA). Mx1 promoter sequence-modified Cre recombinase gene (Mx1-Cre, stock number: 003556) were from the Jackson Laboratory (Bar Harbor, Maine USA). After a mating of these two strains and a second mating of their progeny, mice that were homozygous for the HSF1 floxed allele and also carried the Mx1-Cre transgene were generated (Mx1-Cre\(^{+}\)HSF1\(^{loxp/loxp}\)). Mx1-Cre\(^{+}\)HSF1\(^{loxp/loxp}\) mice were then backcrossed to the Cre\(^{-}\)HSF1\(^{loxp/loxp}\) 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 poly deoxyinosinic/deoxycytidylic acid (pIpC) every 3 days for a total of three injections. Mx1-Cre\(^{+}\)HSF1\(^{loxp/loxp}\) mice that had received injections of pIpC were hereafter referred to as HSF1\(^{-/-}\) mice. Cre\(^{-}\)HSF1\(^{loxp/loxp}\) mice that had received injections of pIpC served as controls and were referred to as HSF1\(^{+/+}\) 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’-AAGCATAGCATCCTGAAAGAGGTAC-3’, and 5’-GTTGTGGTCAGCTCCTGTC-3’, which generated a 482 bp product in floxed
allele, 432 bp product in wild type, and 324bp in knock-out allele. Wild-type male and female C57BL/6 mice, 8-12 weeks old (18-26g), were purchased from Shanghai SLAC laboratory animal Co. LTD (Shanghai, China). A total of 456 male mice and 552 female mice were used in this study.

The procedures used were as humane as possible. 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 NIH 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: SYKX-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 an hour from 4h to 12h after the GalN/LPS injection, then every 12 hours till the 7th day after intoxication. No deaths were observed after the 3rd day, so mice that survived over 72h were considered to survive indefinitely. If an animal was considered possibly morbid, the condition of the animal was monitored every 15 min. 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 7th 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 1 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 (Whatman). After blocked with LI-COR blocking buffer, blots were incubated with anti-Hsp70 (1:500, #4872, Cell Signaling Technology, Inc.), anti-Cleaved Caspase 3 (1:1000, #9664, Cell Signaling), anti-GSK3β (1:1000, ab32391, Abcam), anti-GSK3β pSer9(1:500, ab131097, Abcam), anti-GSK3β pTyr216(1:500, ab75745, Abcam) and anti-β-actin antibodies (1:2000, Santa cruz biotechnology, inc.). After incubation with IRDye800 secondary antibodies (1:10000; LI-COR Biosciences), membranes were washed again in TBS/0.05% Tween 20 for 3 times. 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, 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 (Product Number
78833, Pierce Biotechnology, Inc., USA.), supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche, Indianapolis, IN). 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 (LI-COR Biosciences, Lincoln, NE). Membranes were then incubated with the same blocking solution containing rabbit polyclonal primary antibodies against HSF1 (1:1000, #4356, Cell Signaling Technology, Inc.) and phospho-HSF1 (pSer303) (1:500, ab47369, Abcam). After washing, membranes were incubated at room temperature for 1 h in TBS/0.05% Tween 20 buffer with the IRDye800 secondary antibodies and blots were developed as described above. All values were normalized to a loading control TATA binding protein (TBP, 1:2000, ab818, Abcam) and expressed as 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 alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with a standard clinical automatic analyzer (Siemens Dade behring dimension xpand).

**Liver Histopathology.** Hepatic samples were fixed in 10% neutral buffered formalin overnight, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). For histological analysis, sections were evaluated in a blinded manner by a pathologist. At least 3 fields per section were evaluated.

**Drugs and Experimental Design.** Bicyclol (from Beijing Union pharmaceutical
Plant, Beijing, China) was suspended in phosphate buffered saline (PBS) to produce injectable suspensions and was administered by gavage (300mg/kg every time for three times) in 24 hours. One hour after the last administration, the mice were subjected to intraperitoneal injection of GalN (700mg/kg, Sigma Chemical Co., St Louis, USA) and LPS (10μg/kg, from E. coli serotype 055:B5, Sigma) to induce acute liver failure. Additional animals in control groups received vehicle (PBS) orally, followed by GalN/LPS injection. In other groups, carbon tetrachloride (CCl4, from Sigma, 0.4% diluted in olive oil) was administered intraperitoneally at a single dose of 10 ml/kg at 1 h after the last administration of bicyclol. Some mice received acetaminophen (APAP, from Sigma, dissolved in PBS) intraperitoneally at a single dose of 300 mg/kg at 1 h after the last administration of bicyclol. In separate groups, bicyclol was administered once (300mg/kg) at 1 h after the injection of GalN/LPS or APAP. Dosage of bicyclol was used in accordance with previously published work (Bao and Liu, 2009; Wang and Li, 2006). 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 till the closure of skin wound. After anesthesia was induced, both testes were exposed and sectioned. Analgesia used was 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 skin wound. All 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 (Ts, 12.5mg in 0.1 ml per mouse, Xianju pharmaceutical Co., Ltd., Zhejiang China) by subcutaneous injection (s.c.) in the neck, and 14 days later, the mice were subjected to bicyclol treatment and GalN/LPS intoxication. Anastrozole (Ana, 25μg/mouse, Sigma) was administered s.c. once daily for consecutive 14 days, and followed by GalN/LPS treatment. Some female mice were treated with glycogen synthase kinase 3β (GSK3β) inhibitors. Lithium chloride solution (LiCl, from Sigma, 150mg/kg), SB216763 (from Sigma, dissolved in DMSO, 10mg/kg), or Tideguslib (from Cayman Chemical, dissolved in DMSO, 10mg/kg) were administered intraperitoneally for three times (at the same time point with bicyclol) in 24 hours, followed by GalN/LPS intoxication.

**Statistical Analysis.** All values were reported as the means ± standard deviation (SD). Data were analyzed with a one-way ANOVA with subsequent Student-Newman-Keul’s test or Student’s t-test where applicable. Statistical significance was set at P < 0.05.
Results

There Was a Great Sex Difference in Bicyclol-Induced Hepatoprotection. In the study of bicyclol-induced hepatoprotection, we employed 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 (700mg/kg)/LPS (10ug/kg). Without bicyclol treatment, death of mice (male or female) occurred at 5.5 h and all mice died within 2 hours thereafter. Bicyclol treatment led to a survival rate of over 90% in male mice, but show few protective effects in female mice (Figure 1A). Blood aminotransferases (ALT and AST) increased in female mice despite the treatment of bicyclol, and all of them died within 10h, thereby precluding further time-course studies (Figure 1, C and D). It is worth notice that the elevation of serum ALT was not very significant, and was not comparable with AST, even at 9 h after the intoxication and the mice were going to die within minutes. The macroscopic and microscopic pathological findings were consistent and shown in Figure 1, B and E. The sex difference remained when bicyclol was given after GalN/LPS intoxication (Supplemental Figure 1), or in other liver injury models such as acetaminophen (Supplemental Figure 2) and carbon tetrachloride (Supplemental Figure 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; Bao and Liu, 2009) have demonstrated that bicyclol
induces hepatic heat shock proteins (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 in 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 Figure 2. In line with the serum and histological findings, bicyclol induced Hsp70 expression in male livers but failed to do so in females. Cleaved caspase3, 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 (Figure 3 and Figure 1E). We next administered testosterone (Ts) to female mice, and subjected the mice to GalN/LPS intoxication two weeks later. We also explored the effect of anastrozole (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 (Figure 4 and Figure 1E).
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, first of all we employed 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 employed 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-CreHSF1loxp/loxp mice (HSF1−/−) and Mx1-CreHSF1loxp/loxp littermates (HSF1+/+) were subjected to injections of an interferon inducer pIpC and the successful disruption of the target gene was confirmed by immunoblotting analysis (Figure 5A). Although bicyclol showed remarkable protection in HSF1+/+ control mice, it failed to induce Hsp70 expression and protect against liver failure in HSF1−/− mice (Figure 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 Glycogen Synthase Kinase 3B (GSK3B) 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 Figure 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 (Figure 6B). SB216763 and tideglusib (both are GSK3β inhibitors, Figure 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 results above 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 male and females, liver samples were subjected to immunoblotting analysis using two phosphospecific GSK3β antibodies to probe inactivated phosphorylated serine 9 and activity-enhanced phosphorylated tyrosine 216, respectively. As shown in Figure 7, GSK-3β was constitutively active in livers of both sexes. Bicyclol
treatment inhibited GSK-3β activity in male livers. However, inhibition of GSK-3β 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 results above suggested the sexual dimorphism in bicyclol-induced hepatoprotection was based on bicyclol-induced sexual dimorphism in hepatic GSK-3β activity. If that was true, GSK3β inhibitors and bicyclol should have a synergistic effect in females. So we used three different GSK3β inhibitors, LiCl, SB216763 and tideglusib, which were administered with bicyclol to test the hypothesis. Although these drugs themselves had few effects on GalN/LPS-induced liver damage, anyone of the three produced a remarkable synergistic effect with bicyclol in the induction of hepatic Hsp70 expression and hepatoprotection in females (Figure 8).
Discussion

To protect the liver from various types of liver injury remains to be a major challenge of pharmacotherapy, considering that impairment of liver is a worldwide problem resulted from a lot of etiologies. The fact that the number of hepatoprotective drugs successfully used in the clinical practice is actually very limited accent the clinical importance of bicyclol, a synthetic hepatoprotectant which has been demonstrated to protect from hepatotoxicant-induced, immune-related or ischemia-mediated liver injury. Moreover, in clinical trials and application bicyclol shows 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 (Sun et al., 2012; Zhu et al., 2006). The reported experimental and clinical results are encouraging to conduct more extensive comparative experimental studies with bicyclol, to dissect its molecular basis, and if possible, to improve its clinical effects.

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 serum ALT level was greatly reduced. This was true in all the types of liver damage models we tested (Figure 1, Supplemental figure 1, 2 and 3). Such contradictory outcome 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 low testosterone levels in men occur with increasing age, and are frequently observed in primary care setting, particularly in patients with obesity, metabolic syndrome and acute inflammation (Schneider et al., 2009). All 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 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 the clinical practice.

One of the major findings of this study is that bicyclol induces a 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 the 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 and but not in estradiol-treated rats (Papazosomenos and
Papazozomenos, 2008). 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; Bao and Liu, 2009) have suggested that HSF1 was the key molecular mediator of the pharmacologic effects of bicyclol. In the current study, by using HSF1-/- mice, we are 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 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 Figure 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 (Figure 6). The schematic outline as to how bicyclol and testosterone influenced the heat shock response in the liver was presented in Figure 9.

It was reported (Papasozomenos et al., 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 required 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; 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 of 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
studying 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 and Jianjun Zhang.

Conducted experiments: Chen, Jianjian Zhang, Han, and Dai.

Contributed new reagents or analytic tools: None.

Performed data analysis: Kong, Xu and Xia.

Wrote or contributed to the writing of the manuscript: M. Zhang and Jianjun Zhang.
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Footnotes:

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Financial Disclosure

The authors disclose no conflict of interest.
**FIGURE LEGENDS**

**Figure 1. The sex difference in bicyclol-induced hepatoprotection.** Male or female mice were orally administered with bicyclol (Bic) or PBS, followed by intraperitoneal injection of GalN/LPS. (A) Survival of mice after intoxication (n=20 per group). Bicyclol treatment led to a significant survival advantage in male mice by Kaplan-Meier analysis (log-rank test, P<0.05 between Bic/male and other groups). (B) The gross appearance of mouse livers at 6h (PBS groups) or 8h (Bic groups) after GalN/LPS intoxication. Only bicyclol-treated male livers had a relatively normal gross appearance. Serum ALT (C) and AST (D) concentrations at different time points after intoxication were shown (n=4 per group). * p<0.05 between PBS- and Bic-treated groups; ** p<0.05 between Bic-treated females and males. (E) Representative HE-stained sections from normal livers and post-intoxication livers harvested at 6h (PBS group) or 8h (Bic group) (original magnification, ×200). The livers from PBS-treated mice were harvested at 6h because the mice wouldn’t survive after that. Bic-treated livers were harvested at 8h after intoxication, because hepatic damage in females wouldn’t become obvious until then.

**Figure 2. The sex difference in hepatic Hsp70/Caspase 3 expressions after bicyclol-pretreated intoxication.** (A) Representative immunoblotting results from post-intoxication liver samples. (B-C) Quantitative analyses of the relative levels of protein expression of Hsp70 and active Caspase 3 vs β-actin. “Con” was the control samples from mice that received Bic/PBS treatment but were sacrificed before GalN/LPS intoxication. The mean value obtained from PBS/female/con group was
arbitrarily defined as 1. There were 4 mice at every time point in each group and data were expressed as mean±SD. *P<0.05 between Bic/male and other groups; **P<0.05 between Bic-treated males and females; #P<0.05 between PBS- and Bic-treated groups.

Figure 3. Bicyclol lost hepatoprotective effect in castrated males. Male mice were subjected to castration, followed by bicyclol 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 post-intoxication livers harvested at 6h (PBS group) or 8h (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 4 mice in each group and data were expressed as mean±SD. *P<0.05 vs other groups.

Figure 4. The effect of testosterone (Ts) or anastrozole (Ana) on bicyclol-induced hepatoprotection in females. Female mice were pretreated with Ts or Ana, followed by bicyclol 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 vs the single agent-treated groups). (B) Representative immunoblotting results of protein expression of Hsp70 and active Caspase 3 in livers harvested at 6h (PBS
groups) or 8h (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 4 mice in each group and data were expressed as mean±SD. *P<0.05 vs the single agent groups.

**Figure 5.** The capacity of bicyclol 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 co-detection of TBP was performed to assess equal loading (n = 4 for each group). HSF1 protein bands were then quantified and normalized to TBP. Data were expressed as mean±SD. The results from Cre-negative control (HSF1+/+) mice were arbitrarily defined as 10. *P<0.05 vs Cre-negative controls. (B) Survival of mice after intoxication (n=10 per group). Bicyclol treatment didn’t 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 8h 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 4 mice in each group and data were expressed as mean±SD. *P<0.05 vs HSF1+/+ mice. (E) Representative HE-stained sections from post-intoxication livers harvested at 6h (PBS groups) or 8h (Bic groups). (original magnification, ×200).
Figure 6. The effect of bicyclol on nuclear HSF1 accumulation and phosphorylation (Ser303) in male and female livers. (A) Bicyclol was administered by gavage (300mg/kg) every time for three times in 24 hours. One hour after the last administration, the livers were harvested and nuclear total HSF1 and phosphorylated HSF1 (Ser303) levels were evaluated by western-blot analysis and co-detection of TBP was performed to assess equal loading. (B) HSF1 protein bands were quantified and normalized to TBP. Phosphorylated HSF1 protein bands were quantified and normalized to total HSF1. Bicyclol induced nuclear HSF1 accumulation in both male and female livers, but increased phosphorylated HSF1 (Ser303) level was observed only in females. The mean value obtained from PBS-treated male mice was arbitrarily defined as 1. There were 4 mice in each group and data were expressed as mean ± SD. * P<0.05 vs PBS-treated mice. ** P<0.05 vs Bic-treated females. (C) Administration of Ts to females attenuated bicyclol-induced HSF1 (pSer303) level. (D) pHSF1 protein bands were quantified and normalized to HSF1. The mean value obtained from Bic-treated male mice was arbitrarily defined as 1. There were 4 mice in each group and data were expressed as mean ± SD. **P<0.05 vs Bic-treated female mice. (E) Administration of GSK3β inhibitors (SB216763 and tideglusib) to females attenuated bicyclol-induced HSF1 (pSer303) level. (F) pHSF1 protein bands were quantified and normalized to HSF1. The mean value obtained from Bic-treated female mice was arbitrarily defined as 10. There were 4 mice in each group and data were expressed as mean ± SD. ** P<0.05 vs Bic-treated females.

Figure 7. The effect of bicyclol and Ts on hepatic GSK3β activities. (A) Some
mice had been pretreated with Ts two weeks before. Bicyclol was administered to male and female mice for three times in 24 hours. One hour after the last administration, the livers were harvested and GSK3β (total, pSer9, pTyr216) levels were evaluated by western blot analysis and co-detection of β-actin was performed to assess equal loading (n=6 for each group). (B) pGSK3β bands were quantified and normalized to total GSK3β. The statistical significance was indicated. * p>0.05 vs females without treatment.

Figure 8. The effect of GSK3β inhibitors (LiCl, SB216763 and tideglusib) on bicyclol-induced hepatoprotection in females. Female mice were treated with either 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 Bic as compared with Bic/DMSO-treated mice (log-rank test, P<0.05). GSK3β inhibitors themselves didn’t 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 6h (PBS group) or 8h (Bic group) after intoxication. (C) Protein bands were quantified and normalized to β-actin. There were 4 mice in each group and data were expressed as mean±SD. *P<0.05 vs the single agent groups. (D) Representative HE-stained sections from post-intoxication livers harvested at 6h (PBS groups) or 8h (Bic groups) (original magnification, ×200).

Figure 9. Schematic outline as to how bicyclol and testosterone influence the heat shock response in the liver. When bicyclol is administered to females, the
constitutively activated GSK3β phosphorylates HSF1 at serine 303, thus inactivates it and prevents the heat shock response. By contrast, when bicyclol is administered to males or administered together with testosterone, it not only increases nuclear HSF1 protein level, but also inactivates GSK3β by phosphorylating serine 9 and dephosphorylating tyrosine 216. Without the catalyzing activity of GSK3β, ser303 of HSF1 is dephosphorylated, resulting in the full activation of HSF1 and heat shock response.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

bicyclol: females

activated GSK3β

MSGRPRTTSSFAES...PNVSYICSR...

inactivated HSF1

...VRVKQEPPSPPHSPRVLE...

Heat Shock Transcription

bicyclol: males

inactivated GSK3β

MSGRPRTTSSFAES...PNVSYICSR...

activated HSF1

...VRVKQEPPSPPHSPRVLE...

Hsp70, Hsp27...

bicyclol+Ts: females

activated GSK3β

MSGRPRTTSSFAES...PNVSYICSR...

inactivated HSF1

...VRVKQEPPSPPHSPRVLE...

Heat Shock Transcription