Effects of bicyclol on the activity and expression of CYP450 enzymes of rats after partial hepatectomy

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Abstract: The present study was performed to investigate the effect of bicyclol on hepatic microsomal cytochrome P450 (CYP) activity, as well as gene and protein expressions in rats after partial hepatectomy (PH). Bicyclol (300 mg·kg^{-1}) was given to rats subjected to 70% hepatectomy three times before operation. At 6 and 48 h after PH, blood and liver tissue samples were collected for the measurement of serum alanine aminotransferase (ALT), hepatic microsomal malondialdehyde (MDA) and total hepatic CYP content. The activities of four CYP isozymes were detected with liquid chromatography-mass spectrometry (LC-MS) and the gene and protein expressions were determined by RT-PCR and Western blotting assay. As a result, bicyclol pretreatment markedly inhibited the elevation of serum ALT and hepatic microsomal MDA, and prevented the decrease of total hepatic CYP content in PH rats. In addition, bicyclol significantly attenuated the reduction of CYP2C6 activity and mRNA expression, as well as the reduction of CYP2C11 activity in PH rats. Bicyclol can inhibit the decrease of CYP3A1/2 activity, and up-regulate the mRNA and protein expressions of CYP3A1 and CYP2E1. These results showed that bicyclol pretreatment might ameliorate abnormality in CYP450 isoforms during liver regeneration after PH, and this protective effect was likely due to its anti-oxidative property and enzyme induction.

Key words: hepatectomy; bicyclol; liver; antioxidant; cytochrome P450

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双环醇对肝脏部分切除大鼠 CYP450 酶活性和表达的影响

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摘要: 本研究考察双环醇对肝脏部分切除 (PH) 后大鼠肝脏微粒体细胞色素 P450 (CYP) 活性、基因和蛋白表达的影响及相关机制。大鼠 PH 前灌胃给予双环醇 (300 mg·kg^{-1}) 3 次, PH 后处死大鼠, 取其血清和肝组织进行检测, 依次测定血清谷丙转氨酶 (ALT)、肝微粒体丙二醛 (MDA) 和肝脏总 CYP 含量、4 种 CYP 同工酶活性、基因和蛋白表达。结果显示, 双环醇可显著抑制 PH 大鼠血清 ALT 和肝微粒体 MDA 的升高, 抑制肝脏总 CYP 含量的减少, 抑制 CYP2C6、2C11 活性和 mRNA 表达的下降, 明显抑制 CYP3A1/2 活性的下降, 并上调 CYP3A1 和 2E1 的 mRNA 和蛋白表达。结果表明, 双环醇对 PH 大鼠肝脏 CYP450 酶及部分同工酶活性和表达的改变有明显改善作用, 其作用机制可能与其抗氧化作用和酶诱导作用密切相关。

关键词: 肝切除; 双环醇; 肝脏; 抗氧化剂; 细胞色素 P450

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Cytochrome P450 (CYP) enzymes play an important role in biotransformation of xenobiotics and endobiotics, such as the synthesis of steroid hormones and prostaglandins, activation of vitamin D3 and bile acids, and metabolism and detoxication of drugs[1, 2]. Beside inducers and endogenous regulatory molecules, there are some pathophysiological changes (viruses, cirrhosis, toxic agents) that can influence CYP enzymes activities. It has been reported that hepatic total CYP content after partial hepatectomy (PH) was reduced in rats[3, 4]. However, it was still unclear that CYP isozymes involving in drug metabolism were differently changed in PH.

Bicyclol is a synthetic anti-hepatitis drug for the treatment of chronic hepatitis B patients in China[5]. Previous studies have demonstrated that bicyclol markedly protected against experimental liver injury induced by certain toxins such as carbon tetrachloride, *D*-galactosamine and concanavalin A, and ischemia reperfusion[6−9]. The hepatoprotective mechanism of bicyclol was associated with its antioxidation, regulation of cytokine secretion, and inhibition of apoptosis induced by immunological injury[6]. Additionally, the effect of bicyclol on metabolic enzymes, especially hepatic CYP activity, has been reported. After multiple administrations of bicyclol, CYP2E1 activity in rats was mildly induced, while the activities of CYP2C and 2D were inhibited to certain extent[10].

The purpose of the present studies is to investigate the changes of CYP isozymes at activity, mRNA and protein expression levels, and the effect of bicyclol on CYP isozymes in rats after PH.

Materials and methods

Chemicals Bicyclol was kindly provided by Beijing Union Pharmaceutical Plant (China) and its purity is more than 99%. Trizol was obtained from BioDev Tech Co., Ltd. (China). Alanine aminotransferase (ALT) assay kit was purchased from Beijing Chemical Plant (China). Coomassie blue assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (China). Reverse transcriptional-polymerase chain reaction (RT-PCR) kit was obtained from Takara Biotechnology Co. (Japan). CYP2E1 and 3A antibodies were obtained from Abcam plc.322 Cambridge Science Park (UK) and Santa Cruz Biotechnology (USA), respectively. The compounds below were all purchased from Sigma (USA): diclofenac, mephenytoin, chlorzoxazone, 4-hydroxydiclofenac, 4-hydroxymephenytoin, 6-hydroxychlorzoxazone, 1-hydroxymidazolam and NADPH. Midazolam was obtained from National Institute for the Control of Pharmaceutical and Biological Products (China). Other chemicals were of analytical grade and obtained from the local market.

Animals Male Sprague-Dawley rats weighing 220 to 240 g were obtained from the Beijing Vital River Experimental Animal Co., Ltd. The animal study protocol was in compliance with the guidelines of China for animal care, which was conformed to the internationally accepted principles in the care and use of experimental animals.

Partial hepatectomy model Under anesthesia with 10% chloral hydrate (0.3 $g \cdot kg^{-1}$, intraperitoneally), 70% PH was performed by the method of Higgins and Anderson[11]. In brief, through a midline incision, the left and median liver lobes (corresponding to 70% of liver) were excised after placement of a 4-0 suture ligature on the corresponding pedicle. And then the peritoneal cavity was closed in two layers. At 6 and 48 h after PH, the animals were sacrificed by exsanguination, and then blood and liver tissues were collected for further analysis. To avoid variations due to the circadian rhythms, the operation was performed between 9：00 and 12：00.

Animal treatment Three groups of male rats were included in the study: sham-operated (SH) group, in which an incision was made, and the liver was manipulated, but not ligated (n = 6); PH group, in which rats were subjected to 70% partial hepatectomy as described above (n = 10); bicyclol-pretreatment (By) groups, in which rats were given bicyclol (300 $mg \cdot kg^{-1}$, suspended in 0.5% sodium carboxymethylcellulose) orally for three times in two consecutive days, and the operation was performed at 1 h after the last administration (n = 10). Meanwhile, SH and PH groups received an equivalent volume of vehicle as control.

Preparation of liver microsomes Rat livers were homogenized in 50 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 7.4) containing 200 $mmol \cdot L^{-1}$ sucrose. The homogenates were centrifuged at 10 000×g for 20 min. The resulted supernatant was further centrifuged at 105 000×g for 60 min. Then the pellet was resuspended with 50 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 7.4) containing 250 $mmol \cdot L^{-1}$ sucrose. All procedures were carried out at 4 ℃. Protein concentrations were determined using a Coomassie blue assay kit. Microsomal fractions were stored at −80 ℃ for further analysis.

Assay for CYP enzyme activity Total CYP

contents were quantified by the method of Omura and Sato[12]. The activities of various CYP isozymes were determined by liquid chromatography-mass spectrometry (LC-MS) methods after microsomal incubations as reported previously[13, 14]. In brief, the incubation mixture contained 1 mg·mL^{-1} microsomal proteins, 1.2 mmol·L^{-1} NADPH, 50 mmol·L^{-1} Tris-HCl (pH 7.4), 100 μmol·L^{-1} mephenytoin or 20 μmol·L^{-1} diclofenac, or 100/20 μmol·L^{-1} chlorzoxazone/midazolam in a final volume of 500 μL. The reaction was initiated by addition of NADPH after 5 min preincubation at 37 ℃. After a given incubation time (10 or 20 min), the reaction was terminated by adding 500 μL of cold acetonitrile. Then the samples were centrifuged at 14 000×*g* for 5 min, the supernatant was transferred to a disposable vial in an autosampler and 5 μL was injected for LC-MS analysis.

The LC analysis was carried out with a Zorbax SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm, Agilent, USA). The optimum ESI conditions included the nitrogen nebulizer pressure of 40−60 psi, the nitrogen drying gas temperature of 350 ℃ at 7−9 L·min^{-1}, and spray voltage of 4 000−4 500 V. Mobile phase, ion source and multiple reaction monitoring for metabolites of the probe substrates were shown in Table 1.

Determination of serum ALT and hepatic microsomal MDA content Serum ALT levels and hepatic microsomal malondialdehyde (MDA) content were determined by using commercial assay kits according to the standard procedures.

RT-PCR analysis of CYP 450 mRNA expression Total RNA was extracted from liver tissue using TRIzol reagent. cDNA was reverse-transcribed from 0.5 μg of total RNA using a RT-PCR kit. Corresponding primer sets for PCR were shown in Table 2, and the final volume of reaction was 40 μL. The samples were loaded into a thermal cycler after determining the optimal number of cycles. For each gene, the final cycle was followed by extension at 72 ℃ for 10 min. RT-PCR products were subjected to electrophoresis using 1.2% agarose gel, and then the bands were visualized with ethidium bromide and analyzed by Pro31 software. Results were expressed as ratios relative to β-actin (density of PCR product/density of β-actin).

Western blotting analysis of CYP2E1 and CYP3A protein expression Hepatic microsomal proteins (30 μg) were separated by 12% SDS-PAGE and transferred to PVDF membrane. Bands were then immunologically detected by specific primary antibodies to CYP2E1 (ab28146), CYP3A (sc-25845) or β-actin (sc-1616), and followed by incubation in horseradish peroxidase-conjugated anti-rabbit IgG antibody. Finally, the immunoreactive bands were visualized by ECL Western blotting detection system.

Statistical analysis All results expressed as $\bar{x} \pm s$ were analyzed by one-way analysis of variance (ANOVA) with SPSS 11.0 statistical software package. The differences between means were analyzed by Student-Newman-Keuls (SNK) test for multiple comparisons. P value of less than 0.05 was considered statistically significant.

Results

1 Effect of bicyclol on the elevation of serum ALT level in PH rats

To assess the protective effect of bicyclol on hepatic injury induced by PH, serum ALT detected in rats. The results showed that serum ALT was increased 0.7 times and 3.5 times compared with sham-operated rats (190.8 ± 12.3 U·L^{-1}, 54.1 ± 3.9 U·L^{-1}) at 6 and 48 h in rats after PH. Pretreatment with bicyclol markedly suppressed the elevations of serum ALT (146.4 ± 26.5 U·L^{-1}, 75.7 ± 9.2 U·L^{-1}) in PH rats, which was constant with the previous study[15].

Table 1 CYP enzymes, substrate concentration, mobile phases, ion sources and multiple reactions monitoring for probe substrates metabolites

Enzyme	Substrate	Concentration /μmol·L^{-1}	Metabolite	Mobile phase	Ion source	Precursor (*m*/*z*)	Product (*m*/*z*)
CYP2C6	Diclofenac	20	4-Hydroxydiclofenac	Acetonitrile:water (50∶50, 0.1% formic acid)	ESI (+)	312	268
CYP2C11	Mephenytoin	100	4-Hydroxymephenytoin	Methanol:water (45∶55, 0.1% formic acid)	APCI (+)	235	150
CYP2E1	Chlorzoxazone	100	6-Hydroxychlorzoxazone	Acetonitrile:water (50∶50, 0.005% ammonia water)	ESI (−)	184	148
CYP3A1/2	Midazolam	20	1-Hydroxymidazolam		ESI (+)	342	203

Table 2 Polymerase chain reaction primer sets for CYP isozyme

Gene		Primer sequence (3'–5')	Size/bp	Annealing temperature/℃	PCR cycle No.
CYP2C6	Forward	CGGGAAGTCATACGACATTAGC	759	61	20
	Reverse	GCAGAGAGGCAAATCCATTG			
CYP2C11	Forward	CTGCTGCTGCTGAAACACGTG	248	58	25
	Reverse	GGATGACAGCGTACTATCAC			
CYP2E1	Forward	CTCCTCGTCATATCCATCTG	473	58	22
	Reverse	GCAGCCAATCAGAAATGTGG			
CYP3A1	Forward	ATCCGATATGGAGATCAC	579	58	25
	Reverse	GAAGAAGTCCTTGTCTGC			
CYP3A2	Forward	AGTAGTGACGATTCCAACATAT	252	58	27
	Reverse	TCAGAGGATTCTGTGTTTCCT			
β-Actin	Forward	TGGAATCCTGTGGCATCCATGAAAC	200	62	25
	Reverse	TAAAACGCAGCTCAGTAACAGTCCG			

2 Effect of bicyclol on the elevation of liver microsomal MDA in PH rats

MDA formation is generally used as a biomarker of free radical mediated lipid peroxidation. The data indicated that liver microsomal MDA content increased by 48% at 6 h and observed no significance at 48 h after PH in rats. Bicyclol pretreatment significantly inhibited the elevation of MDA in PH rats (Table 3).

3 Effect of bicyclol on the decrease of total CYP450 content in PH rats

At 6 h after PH, liver microsomal CYP450 content was found no changes in PH rats compared with sham-operated rats. With the increasing of time after PH, total CYP450 markedly decreased by 46% at 48 h after PH. The administration of bicyclol can inhibit the decreasing of liver CYP450 to certain extent (Table 3).

4 Effect of bicyclol on the activities of CYP450 isozymes in PH rats

As shown in Table 4, CYP2C6 activity significantly decreased by 34% at 6 h, and then was further decreased to 73% at 48 h in rats after PH. Bicyclol inhibited the decrease of CYP2C6 activity in the late phase of PH. The activities of CYP2C11 and 3A1/2 were observed no significant changes at 6 h in rats after PH compared with sham-operated rats, however, the activities of both isozymes strikingly decreased by 58% and 36% at 48 h after PH, respectively. Bicyclol showed the protection against the decreasing of CYP3A1/2 activity, and had no effect on CYP2C11 activity. Compared with sham-operated rats, CYP2E1

Table 3 Effect of bicyclol on the formation of MDA and content of CYP450 in liver microsome of PH rats (n = 8). Bicyclol (By, 300 mg·kg^{-1}) was administrated three times before PH in rats. Liver microsomes were prepared at 6, 24, and 48 h after PH. SH: Sham hepatectomy; PH: Partial hepatectomy. $^{\#\#}P < 0.01$ *vs* SH; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* PH

Post-PH time/h	MDA/nmol·mg^{-1} (protein)			CYP 450 content/nmol·mg^{-1} (protein)		
	SH	PH	By	SH	PH	By
6	0.87 ± 0.09	1.22 ± 0.11$^{\#\#}$	0.95 ± 0.11*	0.78 ± 0.10	0.82 ± 0.06	0.81 ± 0.07
24	0.90 ± 0.09	1.14 ± 0.15$^{\#\#}$	0.99 ± 0.06*	0.76 ± 0.10	0.66 ± 0.03	0.75 ± 0.12
48	0.80 ± 0.10	1.03 ± 0.17$^{\#\#}$	0.86 ± 0.09*	0.82 ± 0.12	0.45 ± 0.09$^{\#\#}$	0.79 ± 0.13**

Table 4 Effect of bicyclol on the activities of liver microsomal CYP450 isozyme in PH rats (n = 8). Bicyclol (By, 300 mg·kg^{-1}) was administrated three times before PH in rats. Liver tissues were collected at 6 and 48 h after PH. SH: Sham hepatectomy; PH: Partial hepatectomy. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* SH; $^{**}P < 0.01$ *vs* PH

Group	CYP2C6 /pmol·mg^{-1}(protein)·min^{-1}		CYP2C11 /pmol·mg^{-1} (protein)·min^{-1}		CYP2E1 /pmol·mg^{-1} (protein)·min^{-1}		CYP3A1/2 /pmol·mg^{-1}(protein)·min^{-1}	
	6 h	48 h	6 h	48 h	6 h	48 h	6 h	48 h
SH	695.0 ± 102.2	644.7 ± 85.7	17.8 ± 3.5	24.1 ± 5.8	572.2 ± 80.0	591.9 ± 48.1	142.5 ± 11.7	143.3 ± 19.9
PH	460.9 ± 90.6$^{\#}$	176.2 ± 82.4$^{\#\#}$	16.9 ± 9.4	10.1 ± 1.0$^{\#\#}$	549.3 ± 76.8	485.3 ± 109.1	130.6 ± 31.9	91.1 ± 17.2$^{\#\#}$
Bicyclol	480.9 ± 109.2	414.1 ± 50.9**	14.8 ± 3.8	10.6 ± 5.0	587.7 ± 80.4	550.0 ± 110.3	135.4 ± 19.2	158.1 ± 43.0**

activity was found of no significant changes in all experimental groups.

5　Effect of bicyclol on mRNA expression of CYP isozymes in PH rats

The mRNA expression of CYP2C6 dramatically decreased by 24% and 41% at 6 and 48 h after PH compared with sham-operated rats, which was attenuated by bicyclol pretreatment. Although no significant difference on mRNA expression of CYP3A1 between PH and sham-operated rats was observed, bicyclol pretreatment can strongly enhance the mRNA expression of CYP3A1 at 48 h after PH, while the mRNA expressions of CYP2C11, 2E1 and 3A2 were not changed in PH rats (Figure 1).

6　Effect of bicyclol on the protein expressions of CYP2E1 and CYP3A in PH rats

The protein expression of CYP2E1 in liver microsomes reduced at 6 h after PH, and bicyclol pretreatment can inhibit the down-regulation of CYP2E1 in PH rats. In addition, although no significant difference on CYP3A expression was observed at 6 and 48 h after PH, the expression of CYP3A was markedly up-regulated by bicyclol pretreatment in PH rats (Figure 2, 3).

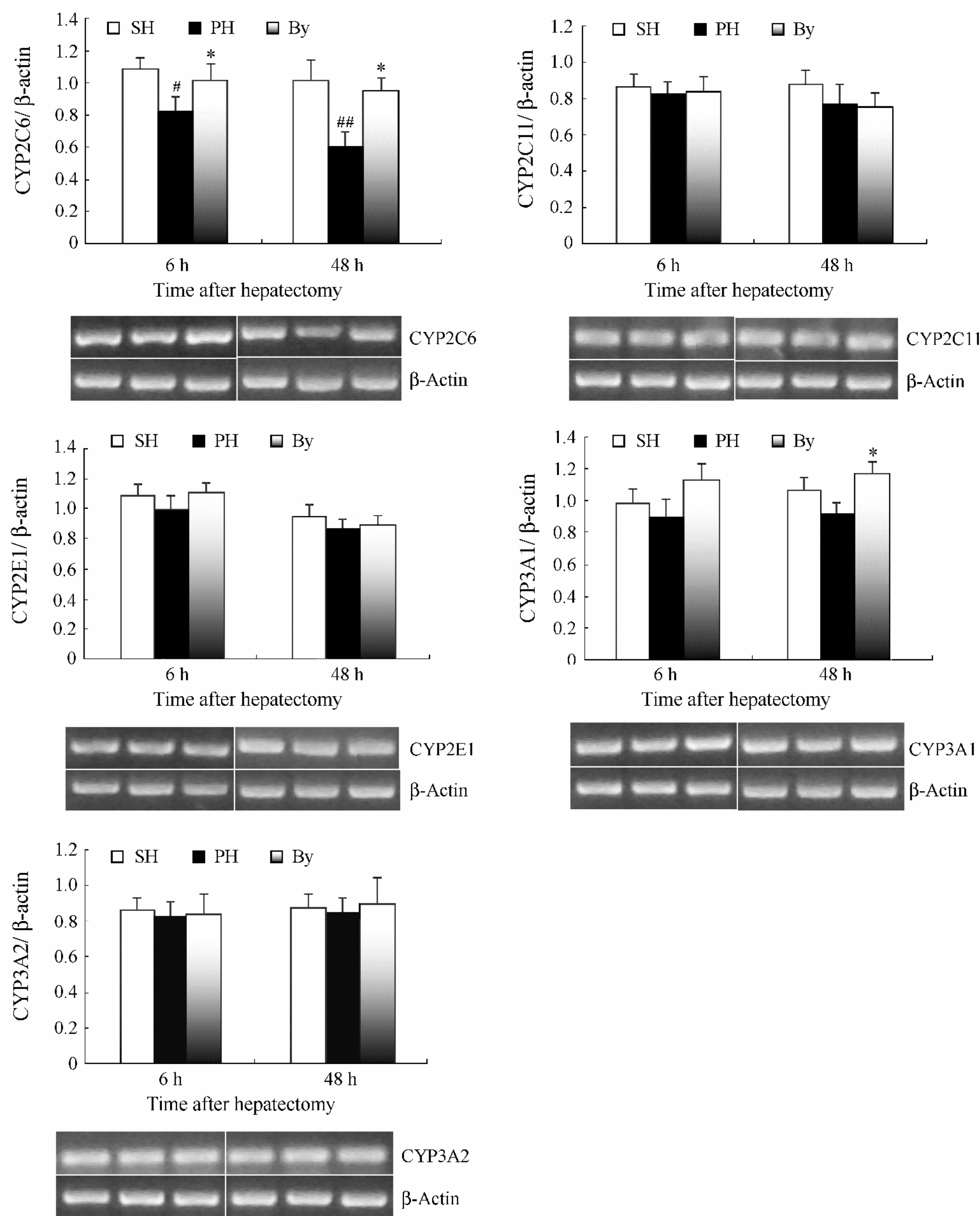

Figure 1　Effect of bicyclol on the mRNA expression of hepatic CYP450 isozymes in PH rats (n = 8). Bicyclol (By, 300 mg·kg^{-1}) was administrated orally three times before PH in rats. SH: Sham hepatectomy; PH: Partial hepatectomy. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* SH; $^{*}P < 0.05$ *vs* PH

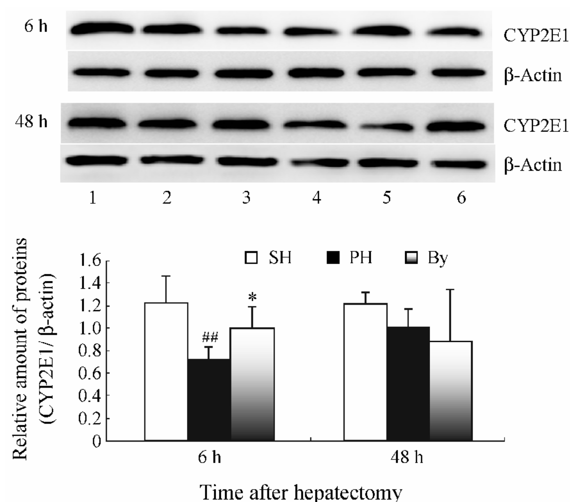

Figure 2 Effect of bicyclol on the protein expression of liver microsomal CYP2E1 in PH rats (n = 4). Bicyclol (By, 300 mg·kg^{-1}) was administrated orally three times before operation. Liver tissues were collected at 6 and 48 h after PH. SH: Sham hepatectomy; PH: Partial hepatectomy. Lane 1, 2: SH; Lane 3, 4: PH; Lane 5, 6: By. $^{\#\#}P < 0.01$ *vs* SH; $^{*}P < 0.05$ *vs* PH

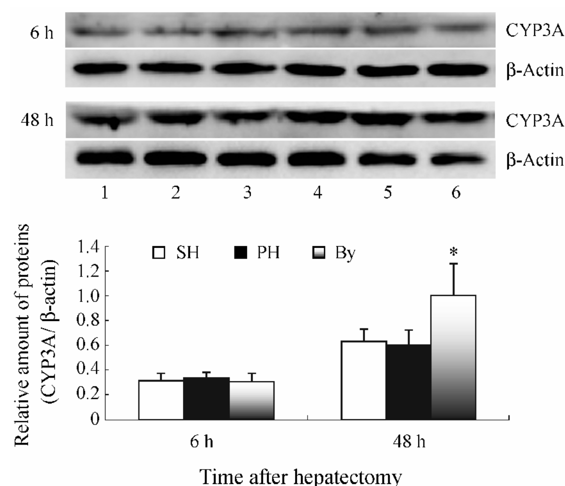

Figure 3 Effect of bicyclol on the protein expression of liver microsomal CYP3A in PH rats (n = 4). Bicyclol (By, 300 mg·kg^{-1}) was administrated orally three times before operation. Liver microsomes were collected at 6 and 48 h after PH. SH: Sham hepatectomy; PH: Partial hepatectomy. Lane 1, 2: SH; Lane 3, 4: PH; Lane 5, 6: By. $^{*}P < 0.05$ *vs* PH

Discussion

Liver is the only organ that exhibits a tremendous ability to regenerate after injury or surgical resection in mammalian body. In general, the recovery of patients after resection of tumors or donation for a living-related liver transplantation depends on the regenerative ability and function of the remnant liver[16]. If liver regeneration is impaired, it can result in the pathogenesis of liver failure or development of fibrosis in humans[17]. Accordingly, a novel pharmaceutical strategy is needed for the protection from liver dysfunction and the enhancement of regenerative capacity. Another study originating from our laboratory has shown that bicyclol pretreatment remarkably enhanced liver regenerative capacity[15]. The results of the present study further demonstrated that bicyclol had a significant protective effect against PH-induced liver injury by inhibition of ALT elevation, attenuation of lipid peroxidation and improvement of the activities and expressions of CYP isozymes.

Many studies have demonstrated that CYP monooxygenase enzymes are susceptible to peroxidative damage[18]. For example, certain chemical agents can stimulate lipid peroxidation, which results in CYP degradation[19], and the purified CYP is destroyed in the presence of various lipid peroxides[20, 21]. Our results have shown that lipid peroxidation occurred in PH rats as evidenced by a significant increase of MDA content in liver tissue[15] and microsomes. Furthermore, there are not many results in the literature concerning the changes in lipid-soluble antioxidant content during liver regeneration, although several measurements for superoxide dismutase (SOD), glutathione (GSH) and peroxidase activities have been made. GSH itself has been reported to be little changed during regeneration, although we have found a significant increase of GSH content in PH rats compared with the sham-operated controls[15, 22, 23]. Bicyclol pretreatment can inhibit the oxidative injury as evidenced by decreasing MDA contents in liver tissue and microsomes, increasing SOD and GSH levels.

CYP, an important factor in drug elimination, are clinically involved in the drug interactions by inhibition and induction[24, 25]. It has been reported that the reduction of CYP content in the damaged liver microsomes can induce the decrease of oxidative activity depending on CYP catalysis, leading to the hepatic drug-metabolizing dysfunction[26]. In the present study, total hepatic CYP content greatly decreased after PH, which can be prevented by bicyclol pretreatment. In addition, CYP family consists of a number of key subtypes, such as CYP1A, 2B, 2C, 2E and 3A, which commonly contribute to the metabolism of drugs in clinics. The change of total CYP content may not completely display that of each isozyme. Thus, we further focused on the changes in the activities and expressions of four major CYP isozymes, and meanwhile investigated the effect of bicyclol on these changes in

PH rats.

Previous studies have indicated that the generation of ROS may damage CYP protein[27]. Our results suggested that the activities of CYP2C6 and 2C11 remarkably reduced after PH, while the mRNA expression of CYP2C6 was also found to be decreased after PH, and no changes in CYP2C11. Bicyclol protected against the decrease of CYP2C6 in both activity and mRNA expression, suggested that it might inhibit these changes by anti-oxidative property. The exact underlying mechanisms, such as protein inactivation, degradation and synthesis needed further investigation.

CYP2E1 is responsible for the metabolism of a large number of low-molecular-weight chemicals, such as aliphatic, aromatic, and halogenated hydrocarbons. Due to its ability to metabolize the compounds, CYP2E1 may be an important determinant of human susceptibility to toxicity and carcinogenicity of industrial and environmental chemicals[28, 29]. Our results showed that CYP2E1 was down-regulated in PH rats, suggesting that the metabolism of the above mentioned chemicals may greatly reduce undergoing hepatectomy, bicyclol can prevent the reduction partially by anti-oxidative property and enzyme induction.

CYP3A was known to catalyze the rate-limiting step in the metabolism and clearance of a large variety of clinical medications, including many pediatric drugs[30]. In the present study, the activity of CYP3A1/2 in PH rats was strikingly diminished after PH, which was prevented by bicyclol pretreatment. But the mRNA and protein expressions of CYP3A1/2 were unchanged after PH, and bicyclol up-regulated the protein expression of CYP3A. The above results, in combination with oxidative injury induced by PH and enzyme induction, suggested that bicyclol can inhibit the reduction of CYP3A1/2 via anti-oxidative property and enzyme induction.

In conclusion, CYP450 content and the activities of four isozymes decreased to certain extent during the liver regeneration after PH, while the individual CYP isozyme was also differentially changed at mRNA and protein levels. Bicyclol pretreatment may ameliorate hepatic drug-metabolizing dysfunction, as indicated by abnormalities in CYP isoforms after PH, and this protection is likely due to its anti-oxidative property and enzyme induction. The complicate regulatory mechanism of bicyclol on related CYPs need to be further investigated.

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