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Chlorpromazine inhibits hepatocyte apoptosis caused by withdrawal of phenobarbital in mice[1]

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KEY WORDS chlorpromazine; verapamil; aspirin; phenobarbital; liver; apoptosis; cell division; DNA

ABSTRACT

AIM: To study the inhibitory effect of chlorpromazine (Chl), verapamil, and aspirin on hepatocyte apoptosis induced by the cessation of phenobarbital (Phe) treatment in mice. **METHODS**: Liver DNA content, ratio of liver weight/body weight, DNA fragmentation, DNA electrophoresis, the end-labeling test (TUNEL), and the morphologic changes of liver cells as indices of liver mass and hepatocyte apoptosis were applied to investigate (1) the kinetic process of hepatocyte proliferation induced by Phe 75 mg·kg^{-1} ip and the regression of hyperplastic liver caused by withdrawal of Phe in mice, (2) the effect of Chl 25 mg·kg^{-1}, verapamil 50 mg·kg^{-1} or aspirin 60 mg·kg^{-1} ip on mouse hepatocyte apoptosis, and (3) the time course of effects of Chl on the regression of liver size and DNA fragmentation content after withdrawal of Phe. **RESULTS**: The process of hepatocyte proliferation and regression induced by administration and withdrawal of Phe in mice consisted of 4 phases: proliferation, plateau, rapid regression, and slow regression phases. In the rapid regression phase, the typic changes of hepatocyte apoptosis were found, which was prevented in early period by the Ca^{2+}-calmodulin antagonist Chl, but not by verapamil or aspirin. **CONCLUSION**: The Ca^{2+}-calmodulin played an important role in the hepatocyte apoptosis caused by withdrawal of Phe.

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INTRODUCTION

Hepatocyte proliferation and apoptosis share common regulatory pathways to maintain the liver *homeostasis*[1,2]. *Fragmeatin*, a granular serine protease that mediates the cytotoxic T-cell-induced apoptosis, activates the P^{34cdc1} kinase that regulates the passage of cells into mitosis[3]. Several genes that are involved in the cell cycle and cellular proliferation, *eg*, c-*jun*, c-*fos*, and c-*myc*, are also involved in apoptosis[4,5]. Therefore, it is necessary to develop an *in vivo* model to study both hepatocyte proliferation and apoptosis. Phenobarbital (Phe) was first considered as a candidate to establish the model, because a non-toxic dosage of Phe stimulated the normal liver growth in various mammalian and after cessation of Phe treatment, the hyperplasia partially regressed and excessive hepatocytes were eliminated by apoptosis[6]. The present study was to investigate the inhibition of chlorpromazine (Chl, a Ca^{2+}-calmodulin antagonist), verapamil (a Ca^{2+} channel blocker), and aspirin (a cyclooxidase inhibitor) on the hepatocyte apoptosis induced by the withdrawal of Phe in mice.

MATERIALS AND METHODS

Reagents Trypsin, RNase, and bovine thymus DNA were purchased from Sigma Chemical Co. TUNEL kit was obtained from Boehringer Mannheim Biochemicals. Chl was produced by Shanghai Tianfeng Pharmaceutical Co, and Phe was produced by Shanghai New Asia Pharmacy Co. All other chemicals were of analytical reagent.

Mice Kunming strain ♂ mice (age 4 - 6 wk, $n = 302$, weight ($20 \pm s$ 2) g, Grade Ⅱ, certificate number 005) were obtained from the Shanghai Experimental Animal Center, Chinese Academy of Sciences. All mice were adapted for 1 - 2 wk to a

room with lights off from 20:00 to 8:00, and food was available from 9:00 to 14:00 with drinking water *ad lib*.

Kinetics of hepatocyte proliferation and regression caused by administration and withdrawal of Phe in mice Mice were randomly divided into 11 groups, 11 – 12 in each. The mice were given ip Phe 75 mg·kg^{-1}·d^{-1} for 7 d. The mice were decapitated and liver was stored at – 20 ℃ for 1 – 2 wk before processing[7]. Other specimens of fresh liver were fixed in formalin and embedded in paraplast. Sections (5 μm) were stained with hematoxylin and eosin.

Effect of Chl, verapamil, and aspirin on hepatocyte apoptosis induced by withdrawal of Phe Mice were randomly divided into 6 groups (10 in each). Five groups were injected ip 0.5 % Phe 75 mg·kg^{-1} for 5 d, while one group was given equal amount of 0.9 % NaCl. After the withdrawal of Phe, 4 groups were injected ip Chl 25 mg·kg^{-1}·d^{-1}, aspirin 60 mg·kg^{-1}·d^{-1}, verapamil 50 mg·kg^{-1}·d^{-1}, or equal amount of 0.9 % NaCl q8h. The mice of negative control group were decapitated immediately. Exactly at 36 h after stopping Phe treatment, the livers were taken. The histologic changes, the ratio of liver/body weight (LW/BW), the content of hepatic DNA fragmentation[7], DNA electrophoresis[8], and the end-labeling test (TdT-mediated X-dUTP nick end labeling, TUNEL) were determined.

Time course of effects of Chl on the regression of liver size and DNA fragmentation content after withdrawal of Phe in mice Mice were randomly divided into 14 groups (8 in each). Eight groups were injected ip 0.5 % Phe 75 mg·kg^{-1} for 5 d and 2 groups as normal control were given equal amount of 0.9 % NaCl. Four groups as negative control were continuously given ip 0.5 % Phe 75 mg·kg^{-1}·d^{-1}. After the withdrawal of Phe, 4 groups were injected ip Chl and the other 4 groups as positive control administered ip 0.9 % NaCl. At 24, 36, 48, and 72 h after the withdrawal of Phe, the livers were taken and the content of hepatic DNA fragmentation was measured[7].

Statistical analysis Data were expressed as $\bar{x} \pm s$, and compared using Dunnett's t test for multiple differences.

RESULTS

Kinetics of hepatocyte proliferation and regression caused by administration and withdrawal of Phe in mice After administered ip Phe in mice, the size of liver grew rapidly and reached the maximum in 3 – 5 d, when the content of hepatic DNA increased by 22.4 % and LW/BW increased by 28.1 %. Thereafter, although Phe was given continuously, the liver size kept stable. Twenty-four hours after cessation of Phe treatment the involution size declined rapidly until 48 h when the involution liver changed into slow regression. The computer curve imitation of DNA content-time relationship was made by Marquardt's method. The theoretical equation: $A_1^{-K_a t} + A_2^{-\alpha t} + A_3^{-\beta t}$, $K_a = 0.6110$, $K_a \cdot T_{1/2} = 1.12$ d, $\alpha = 0.3900$, $\alpha \cdot T_{1/2} = 2.45$ d, $\beta = 0.0173$, $\beta \cdot T_{1/2} = 33.20$ d (Fig 1).

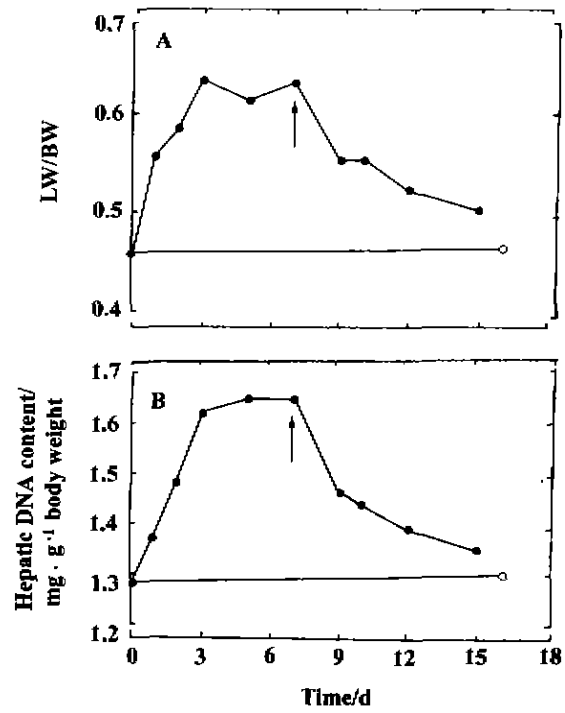

Fig 1. Effect of Phe treatment and Phe withdrawal on liver size and DNA content in mice. A) LW/BW; B) hepatic DNA content. ● treated mice, ○ controls. ▲ withdrawal of Phe treatment.

Effect of Chl, verapamil, and aspirin on hepatocyte apoptosis induced by withdrawal of

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Phe The regression of liver size, the increasing of DNA fragmentation, and apoptotic bodies, which occurred in the positive control mice after withdrawal of Phe as shown in Tab 1 and Fig 2, were not found in the mice treated with Chl, and a negative result of TUNEL assay was presented. However, in the mice given verapamil and aspirin the liver size markedly regressed, DNA fragmentation increased and the apoptotic bodies with a positive reaction of TUNEL assay were found. In our experiments the DNA sample extracted from the liver of positive control mice did not demonstrate the characteristic ladder pattern on gel electfophoresis (Fig 3), which was considered as a marker of the apoptotic mode of cell death.

Tab 1. Effect of Chl, verapamil, and aspirin on regression of liver size and content of hepatic DNA fragmentation after withdrawal of Phe in mice. $n = 10$, $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **negative control.**

Group	LW/BW	Hepatic DNA fragmentation/ $\mu g \cdot g^{-1}$ tissue
Normal control	0.42 ± 0.03^c	50 ± 17
Negative control	0.57 ± 0.03	44 ± 12
Positive control	0.50 ± 0.03^b	77 ± 21^b
Chl	0.58 ± 0.05	40 ± 11
Verapamil	0.52 ± 0.03^b	90 ± 29^c
Aspirin	0.51 ± 0.04^b	75 ± 29^b

Time course of effects of Chl on the regression of liver size and DNA fragmentation content after withdrawal of Phe As in the kinetic studies of hyperplastic liver regression, until 24 h after cessation of Phe treatment the liver size did not regress. At 36 h the content of hepatic DNA fragmentation increased by 109.8 % and LW/BW declined by 11.8 % in the mice as positive control, but did not in the mice treated with Chl. However, at 48 h the content of hepatic DNA fragmentation rose to 164.7 % of negative control value and the liver size regressed by 7.4 % in the mice treated with Chl. At 72 h the inhibiton of Chl on the regression of liver size and the increasing of DNA fragmentation content dissipated (Tab 2).

DISCUSSION

We found that the kinetic process of hepatocyte proliferation and regression caused by administration and withdrawal of Phe in mice consisted of 4 phases: proliferation, plateau, rapid regression, and slow regression phases; and the rapid regression of hyperplastic liver involved hepatocyte apoptosis, which was prevented by the Ca^{2+}-calmodulin antagonist Chl, but not by Ca^{2+} channel blocker verapamil and cyclooxidase inhibitor aspirin.

The time course investigation of effects of Chl on the regression of liver size and DNA fragmentation content showed that only in the early period of rapid regression phase, Chl was available for the inhibiting of hyperplastic liver regression, and this effect was different from that of Phe or cyproterone, stimulus of hepatocyte proliferation. In rats administered Phe to replace cyproterone, the size of hyperplastic liver kept stable and no regression occurred[9]. The results imply that the effect of Chl is to prevent hepatocyte apoptosis induced by withdrawal of Phe rather than to stimulate hepatocyte proliferation. Chl and verapamil prevented

Tab 2. Time course of Chl effects on regression of liver size and DNA fragmentation content ($\mu g \cdot g^{-1}$ tissue) after withdrawal of Phe in mice. $n = 8$, $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **negative control.**

Group	24 h		36 h		48 h		72 h	
	LW/BW	DNA fragmentation	LW/BW	DNA fragmentation	LW/BW	DNA fragmentation	LW/BW	DNA fragmentation
Chl	0.68 ± 0.07	52 ± 12	0.70 ± 0.05	53 ± 13	0.62 ± 0.08	85 ± 22^c	0.57 ± 0.06^c	61 ± 12^b
Positive control	0.66 ± 0.07	46 ± 9	0.60 ± 0.06^b	104 ± 36^c	0.58 ± 0.04^b	78 ± 24^b	0.56 ± 0.05^c	58 ± 19^b
Negative control	0.65 ± 0.06	53 ± 15	0.68 ± 0.05	50 ± 8	0.67 ± 0.05	51 ± 11	0.69 ± 0.07	51 ± 15
Normal control	0.48 ± 0.05^c	52 ± 10					0.50 ± 0.07^c	48 ± 16

Fig 2. End-labeling test of mouse liver tissue. (A) positive reaction shown in positive control group × 400; (B) negative reaction in Chl group × 200. HE stain.

the increase of nuclear Ca^{2+} and DNA fragmentation and nearly abolished biochemical evidence of toxic hepatocyte death induced by acetaminophen[7]. Nicotera *et al*[10] also found that Ca^{2+} was actively transported into hepatocyte nucleus by a calmodulin-dependent pump that utilized ATP as the energy source. These findings support the hypothesis that the loss of Ca^{2+}-regulation is the final common pathway to cell death and Chl exerts its hepatoprotective effect by antagonizing Ca^{2+}-calmodulin. Why Ca^{2+} channel blocker verapamil protects hepatocytes from the toxicity of acetaminophen but does not inhibit the hepatocyte apoptosis caused by the withdrawal of Phe is not known to us yet. Cyclooxidase inhibitor aspirin prevents the synthesis of prostaglandins, the signal transducer of hepatocyte growth factor (HGF). However, aspirin did not effect the hepatocyte apoptosis induced by withdrawal of Phe, suggesting that the association between hepatocyte growth and apoptosis was complicated.

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Fig 3. Electrophoresis of DNA from mouse liver.
a: hepatic DNA from positive control mouse.
b: λgt/Hind Ⅲ.

In the present experiments, the morphologic changes of hepatocyte apoptosis were shown after cessation of Phe treatment, but agarose gel electrophoresis did not reveal a ladder of DNA fragmentation. The contradiction can be explained by the finding that the mean duration of the histologic stages of apoptosis was found to be about 3 h, and within the duration there was a relatively low incidence of cell apoptosis[6].

As mentioned above, Phe mode can be applied to study hepatocyte proliferation and apoptosis, and the Ca^{2+}-calmodulin pathway plays a significant role in the hepatocyte apoptosis induced by withdrawal of Phe.

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氯丙嗪对撤除苯巴比妥钠诱导的小鼠肝细胞凋亡有抑制作用[1]

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关键词 氯丙嗪; 维拉帕米; 阿司匹林; 苯巴比妥; 肝; 细胞凋亡; 细胞分裂; DNA

目的: 研究氯丙嗪(Chl)、维拉帕米和阿斯匹林对撤除苯巴比妥钠(Phe)引起的小鼠肝细胞凋亡有无抑制作用及其时效关系. **方法**: 以肝 DNA 含量、肝/体重比、DNA 片段含量、DNA 电泳、原位 DNA 末端标记试验(TUNEL)及形态学改变为指标, 研究 Phe 75 $mg \cdot kg^{-1} \cdot d^{-1}$ ip 诱导小鼠肝细胞增殖及撤药后增殖肝回落的动态过程, 并观察 Chl 25 $mg \cdot kg^{-1}$、维拉帕米 50 $mg \cdot kg^{-1}$或阿司匹林 60 $mg \cdot kg^{-1}$ ip 对小鼠肝细胞凋亡的影响及其时程效应. **结果**: Phe 诱导小鼠肝细胞增殖及停药后增殖肝的回落由增殖期、平台期、快速回落期和缓慢回落期四个时相组成, 快速回落期是一个典型的凋亡过程, 其早期能被钙调蛋白拮抗剂 Chl 阻断. **结论**: 钙调蛋白在撤除 Phe 引起的小鼠肝细胞凋亡中起重要作用.

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