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Induction of hepatocyte proliferation and prevention of hepatocyte apoptosis by phenobarbital related to local humoral factor in mouse liver[1]

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KEY WORDS phenobarbital; liver; cell division; apoptosis; high pressure liquid chromatography

AIM: To study the association of phenobarbital (Phe) inducing hepatocyte proliferation and blocking hepatocyte apoptosis with local humoral factor in liver. **METHODS:** The ratio of liver/body weight, DNA content, regressive rate of hyperplastic liver, and DNA fragmentation were used to investigate whether the Phe-treated mouse liver extract (PMLE) and PMLE-95 (PMLE heated at 95 ℃ for 30 min) possessed Phe-like effects on mouse liver. Meantime, the effects of pretreatment with trypsin, RNAase, and DNAase on the activity of PMLE-95 were observed, and the differences of components between PMLE-95 and NMLE-95 (normal mouse liver extract, NMLE heated at 95 ℃ for 30 min) were analyzed with HPLC. **RESULTS:** PMLE-95 stimulated hepatocyte proliferation and prevented hepatocyte apoptosis caused by withdrawing Phe in mice, and the activity of PMLE-95 was eliminated after the pretreatment with trypsin. On the chromatograms PMLE-95 had 5 main peaks, while NMLE-95 had only 4 peaks. **CONCLUSION:** The effects of Phe on the liver were mediated by an intrinsic protein or peptide substance produced in response for the stimulation of Phe in mouse liver.

Phenobarbital (Phe) has been used to induce hepatic microsomal enzymes, prevent hepatocyte apoptosis, and promote hepatocarcinogenesis[1-3]. A nontoxic dosage of Phe can stimulate hepatocellular DNA synthesis and proliferation with increased activities of drug-metabolizing enzymes in various mammalians. There are many humoral factors involved in the control of hepatocellular growth. The complete hepatocyte mitogens such as hepatocyte growth factor (HGF) and hepatic stimulator substance (HSS) can strongly stimulate hepatocellular DNA synthesis and mitosis; the comitogens such as insulin and norepinephrine are necessary to regulate the growth of regenerating liver. The aim of the present experiment was to study whether Phe-treated mouse liver extract (PMLE) possessed Phe-like effects on mouse liver, and whether the components of PMLE differed from those of normal mouse liver extract (NMLE) by HPLC analysis

MATERIALS AND METHODS

Reagents and instruments Trypsin, RNAase, and DNAase were purchased from Sigma Chemical Co; HPLC-grade acetonitrile was manufactured by Baxter Healthcare Co, USA; Phe was produced by Shanghai New Asia Pharmacy Co; Other chemicals were of AR. Beckman HPLC system (USA) consisted of a pair of 125 solvent delivery unit, a 168 UV-detector, a 150-466 DX2 personal computer, and an Epson LQ-300 recorder; an analytical column (250 mm × 4.6 mm) was packed with Spherisorb C_{18} 5 μm.

Mice Kunming strain mice (♂, n = 191, age 4 - 6 wk, weighing 18 - 22 g) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade Ⅱ, Centificate № 005). All mice were fasted for 12 h before the livers were taken.

Preparation of mouse liver extracts Thirty mice were randomly divided into 3 groups (10, each). The mice in A and B groups were injected ip 0.5 % Phe 75 $mg \cdot kg^{-1} \cdot d^{-1}$ for 3 d and 7 d, respectively; the mice in C group were given equal amount of 0.9 % NaCl. The mice were killed by cervical dislocation during 7 - 8 AM and the livers were excised, weighed, and flushed with ice-cold 0.9 % NaCl. The hepatic homogenate was prepared in 4 volumes (wt/vol) of 0.9 % NaCl. This homogenate was divided

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into 2 parts: a share was heated at 95 ℃ for 30 min and the other remained at 4 ℃ for 30 min. Then the homogenates were centrifuged at 27 000 × g at 4 ℃ for 20 min. The supernatant was further centrifuged at 100 000 × g for 1 h. The resultant supernatants were collected, after measuring the protein content by Folin-phenol method, lyophilized, and stored at − 80 ℃ until use. Normal mouse liver extract (NMLE) was heated at 95 ℃ (NMLE-95) and PMLE was heated at 95 ℃ (PMLE-95).

Determination of proliferation activity of liver extracts Mice (n = 64) were randomly divided into 8 groups (8, each). The mice as positive control were injected ip 0.5 % Phe 75 $mg \cdot kg^{-1} \cdot d^{-1}$ for 3 d; the mice as negative control received equal amount of 0.9 % NaCl. The other 6 groups were injected ip the extracts 2.5 or 25 $mg \cdot kg^{-1} \cdot d^{-1}$ for 3 d. All mice were killed 24 h after the treatment and the livers were taken for determining the liver/body weight ratio and DNA content by the diphenylamine method[4].

Effects of liver extracts on hepatocyte apoptosis caused by withdrawal of Phe Mice (n = 49) were randomly divided into 7 groups (7, each). The mice as positive control were injected ip 0.5 % Phe 75 $mg \cdot kg^{-1} \cdot d^{-1}$ for 5 d and the mice as negative control were given equal amount of 0.9 % NaCl. The other mice were injected ip 0.5 % Phe 75 $mg \cdot kg^{-1} \cdot d^{-1}$ for 3 d, and then given various hepatic extracts 2.5 or 25 $mg \cdot kg^{-1} \cdot d^{-1}$ for 2 d. At 48 h after withdrawal of Phe the livers were taken for measuring the liver/body weight ratio and DNA fragmentation content[4].

Bioassay of the activity of PMLE-95 preventing hepatocyte apoptosis PMLE-95 was incubated with trypsin 500 $mg \cdot L^{-1}$, RNAase or DNAase 250 $mg \cdot L^{-1}$ at 37 ℃ for 2 h, and then heated at 95 ℃ for 15 min. *In vivo* experiments of the effects of trypsin, RNAase, and DNAase on the activity of the hepatic extract were performed according to the protocol as described above.

HPLC analysis of PMLE-95 and NMLE-95 components PMLE-95 and NMLE-95 were diluted with phosphate-buffered saline (PBS, pH 7.4) into the concentration of protein 0.5 $g \cdot L^{-1}$, and after passing through a 0.2 μm filter, 20 μL of this solution was injected into the liquid chromatograph. The mobile phase was phosphate buffer 10 $mmol \cdot L^{-1}$ (pH 3.5) run at a flow rate of 1 $mL \cdot min^{-1}$, with a linear gradient of acetonitrile from 0 % − 30 %, and λ = 254 nm.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared using Dunnett's modification of t test.

RESULTS

NMLE like PMLE, no matter heated or unheated, dramatically increased liver/body weight and DNA content (Tab 1).

Tab 1. Proliferation activity of liver extracts. n = 8 mice. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* normal control.

Group/$mg \cdot kg^{-1} \cdot d^{-1}$		Liver/BW ratio	DNA content (mg/g BW)
Normal control		0.45 ± 0.04	0.38 ± 0.04
Phe control	75	0.63 ± 0.06^c	0.49 ± 0.05^c
NMLE-95	2.5	0.54 ± 0.02^b	0.42 ± 0.04^b
NMLE	25	0.52 ± 0.03^b	0.43 ± 0.04^b
PMLE-95 (Phe 3 d)	2.5	0.52 ± 0.03^b	0.44 ± 0.04^b
PMLE (Phe 3 d)	25	0.54 ± 0.03^b	0.43 ± 0.04^b
PMLE-95 (Phe 7 d)	2.5	0.54 ± 0.03^b	0.44 ± 0.03^b
PMLE (Phe 7 d)	25	0.52 ± 0.04^b	0.42 ± 0.04^b

Only PMLE-95 inhibited the increment of DNA fragmentation and the regression of hyperplastic liver which occurred after cessation of administering Phe; this preventing action was eliminated by the pretreatment with trypsin (Tab 2, 3).

On the HPLC PMLE-95, induced by Phe for 3 d or 7 d, had 5 main peaks, while NMLE-95 had only 4 peaks. The retention time of the further peak was 6.2 min (Fig 1).

DISCUSSION

Understanding the control of cellular growth has obvious implications for the elucidation of both normal and malignant growth; and the hepatocyte proliferation induced by Phe provides an excellent model with which to study the problem. Because Phe itself is not a mitogenic drug in cell culture, we assume that the hepatocyte proliferation induced by Phe is involved in the local humoral factor(s) in liver,

Tab 2. Effects of liver extracts from mice treated with Phe on hepatic DNA fragmentation and regression. $n = 7$ mice. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* Phe control.

Group	/mg·kg^{-1}·d^{-1}	Liver/BW ratio weight	Regression rate (%)	DNA fragmentation (μg/g liver)
Normal control		0.48 ± 0.05^c		47 ± 11^a
Phe control	75	0.61 ± 0.05		44 ± 12
0.9 % NaCl		0.54 ± 0.05^b	11.5	78 ± 18^b
NMLE-95	2.5	0.56 ± 0.05^b	8.2	73 ± 19^b
NMLE	25	0.55 ± 0.06^b	9.8	73 ± 16^b
PMLE-95	2.5	0.62 ± 0.05^a		41 ± 10^a
PMLE	25	0.54 ± 0.05^b	11.5	76 ± 18^b

Tab 3. Effects of trypsin, RNAase, and DNAase on the activity of PMLE-95. $n = 7$ mice. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* Phe control.

Group	Liver/BW ratio	Regression rate (%)	DNA fragmentation (μg/g liver)
Normal control	0.46 ± 0.03^c		49 ± 16^a
Phe control	0.63 ± 0.04		52 ± 13
0.9 % NaCl	0.54 ± 0.04^b	14.3	83 ± 18^b
Trypsin	0.55 ± 0.03^b	12.7	86 ± 19^c
RNAase	0.64 ± 0.05^a		53 ± 12^a
DNAase	0.62 ± 0.05^a	1.6	50 ± 15^a

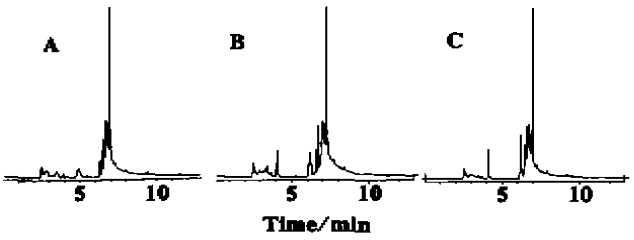

Fig 1. Liquid chromatograms of NMLE-95 (A) and PMLE-95 induced by Phe for 3 d (B) and 7 d (C).

which is produced by administering Phe. If so, the extracts from the hyperplastic liver induced by Phe would possess the effects of Phe-like on the normal liver. The experimental results demonstrated that pharmacologic effects and chromatographic components of PMLE-95 significantly differed from those of NMLE-95. PMLE-95 like Phe was able to stimulate mouse hepatocyte proliferation and block hepatocyte apoptosis, while NMLE-95 was unable to prevent mouse hepatocyte apoptosis in spite of inducing hepatocyte proliferation. The analysis with HPLC showed that there were five main peaks in PMLE-95 but only four in NMLE-95. After the pretreatment with trypsin the activity of PMLE-95 was eliminated, but the pretreatments with RNAase and DNAase did not affect its activity. As mentioned above, our investigations indicate that the induction of hepatocyte proliferation and prevention of hepatocyte apoptosis by Phe are associated with the local humoral factor(s) in mouse liver, and the factor(s) is a protein or peptide substance.

Because Phe does not interfere with the chromatographic peaks of PMLE-95 under the conditions used by us, it is impossible that the difference of HPLC behavior between PMLE-95 and NMLE-95 is caused by Phe itself. The preparing procedures of PMLE-95 are similar to those of HSS[5]; therefore, the heat-labile stimulators such as HGF, EGF, PDGF (platelet-derived growth factor) and insulin are not considered to relate to the activity of PMLE-95. Further work is directed toward the purification and characterization of PMLE-95.

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苯巴比妥诱导小鼠肝细胞增殖和阻断肝细胞凋亡的作用与小鼠肝内局部体液因素有关[1]

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关键词 苯巴比妥; 肝; 细胞分裂; 细胞凋亡;

高压液相色谱法

目的：研究苯巴比妥(Phe)诱导肝细胞增殖和阻断肝细胞凋亡的作用是否与肝内局部体液因素有关. 方法：观察 Phe 诱导增殖的小鼠肝提取物(PMLE)及其加热提取物 (PMLE-95)对小鼠肝有无 Phe 样作用；经胰蛋白酶、RNA 酶或 DNA 酶预处理后，其活性是否消失. 高效液相色谱分析增殖肝与非增殖肝加热提取物(NMLE-95)的组份差异. 结果：PMLE-95 使正常肝细胞增殖，阻断撤除 Phe 引起的小鼠肝细胞凋亡；胰蛋白酶处理后，该作用消失. HPLC 分析，PMLE-95 比 NMLE-95 多一个主峰. 结论：Phe 诱导小鼠肝细胞增殖和阻断肝细胞凋亡的作用与肝内局部体液因素有关，该因素是一种蛋白质或肽类物质.

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Levels of immunoreactive dynorphin A_{1-13} during development of morphine dependence in rats[1]

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KEY WORDS dynorphin; morphine; opioid-related disorders; radioimmunoassay; substance withdrawl syndrome

AIM: To study the relationship between the levels of immunoreactive dynorphin A_{1-13} (ir-dynorphin A_{1-13}) and the degree of morphine dependence. **METHODS:** The levels of ir-dynorphin A_{1-13} in discrete brain regions, spinal cord, and plasma in rats were determined by radioimmunoassay, and the degree of morphine dependence was assessed by scoring withdrawal signs on d 3, d 6, and d 12. **RESULTS:** Morphine injection sc decreased the levels of ir-dynorphin A_{1-13} in spinal cord, pituitary, and plasma. The levels of ir-dynorphin A_{1-13} in hippocampus and hypothalamus were increased. No changes in cortex, midbrain, cerebellum, pons, and medulla were observed. With continuous injection of morphine, withdrawal signs scores were increased on d 6, but there was no difference between the scores of d 6 and d 12. **CONCLUSION:** The changes of the levels of endogenous ir-dynorphin A_{1-13} in pituitary, spinal cord, and plasma were compatible with the degree of morphine dependence.

Dynorphin A_{1-13} can ameliorate the signs of opiate withdrawal and suppress the expression of opiate tolerance in morphine-dependent mice[1,2], rats[3], monkey[4], and heroin addicts[5]. Ir-dynorphin A_{1-13} plays an important role during the induction of tolerance to and dependence on morphine in rats, and it is possible that ir-dynorphin A_{1-13} levels in spinal cord and some brain regions were decreased, so that the administration of exogenous dynorphin A_{1-13} restored the levels and thereby attenuated withdrawal signs. However, the results obtained were unlike[6,7] and during the development of dependence on morphine the dynamic changes of ir-dynorphin A_{1-13} levels in rats and its relationship with the degree of dependence have not ever been reported. The present experiments were to study the relationship between the levels of immunoreactive dynorphin A_{1-13} and the degree of morphine dependence in rats.

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