

Effect of mitoxantrone on DNA polymerase of Ehrlich ascites carcinoma cells¹

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KEY WORDS mitoxantrone; enzyme inhibitors; DNA polymerases; Ehrlich tumor carcinoma; doxorubicin; aphidicolin

AIM: To study the effect of mitoxantrone (Mit) on DNA polymerases of tumor cells.

METHODS: DNA polymerases of Ehrlich ascites carcinoma cells were isolated by phosphocellulose column chromatography. The effects of Mit on DNA polymerase α , β , and γ were detected by method of K Ono.

RESULTS: Mit inhibited DNA polymerase α , β , and γ , IC_{50} values were 11.9, 6.5, and 11.9 $\mu\text{mol} \cdot \text{L}^{-1}$, and K_i 1.86, 2.22, and 2.05 $\mu\text{mol} \cdot \text{L}^{-1}$, respectively. The inhibitory mode of Mit on DNA polymerase α , β , and γ was competitive. **CONCLUSION:** Mit is a strong inhibitor on DNA polymerase α , β , and γ . The inhibitory mode was competition with respect to template DNA.

Mitoxantrone (Mit), a new anthraquinone antitumor drug^[1], mimicked the basic structures of doxorubicin (Dox) and was expected to have less cardiotoxicity, Mit was used to treat the acute leukemia, malignant lymphoma, breast cancer, and the tumors of digestive system.

DNA polymerases (Pol) are key enzymes in the DNA synthesis. At least 5 kinds of Pol were found in eucaryocytes. Pol catalyze the dNTP polymerizing into DNA, and play an important role in ensuring fidelity of DNA replication through their catalytic function, template-specific selection, and 3'-exonuclease activity, which has the function of proofread in DNA synthesis. The 5'-exonuclease activity of Pol would repair damaged DNA. Pol α and δ are responsible for DNA replication, Pol β and ϵ are related to DNA repair, while Pol γ is implicated in the replication of mitochondrial DNA^[2-5].

Due to the closed relationship between Pol and DNA replication, the high activity of Pol was considered as the feature of malignant tumor, and thus Pol become the target of some antitumor drugs. Cytarabine (Cyt), aclarubicin, and Dox are inhibitors of Pol^[6].

This paper was to study the effect of Mit on DNA polymerases of Ehrlich ascites carcinoma (EAC) cells.

MATERIALS AND METHODS

Kunming mice (δ , 6 wk-old, body weight 19-21 g, Grade II) were purchased from Department of Experimental Animals, First Military Medical University (Certificate No 94004). EAC cells were purchased from Institute of Medical Biotechnology, Chinese Academy of Medical Sciences, Beijing, and routinely passaged weekly by inoculating into the body of mouse.

Drugs, reagents, and experimental materials Mit was produced by Zhe Nan Pharmaceutical Factory (Lishui, Zhejiang, lot No 910618); Dox (Hua Ming Pharmaceuticals Co Ltd, Shantou, Guangdong, lot No 910902); activated calf thymus DNA, type XV (Act DNA) (Sigma Co, No 109F-6757); dATP, dCTP, dGTP (Promega Co, lot No 141401, 122302, 122401, respectively); [³H]dTTP (Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, lot No 911213, 814 TBq $\cdot \text{mol}^{-1}$). Aphidicolin was produced by Sigma Co, lot No 31H4040. Phosphocellulose (Whatman Co). Acetylfibrocyn membrane (Siqing Biochemical Factory, Huangyan, Zhejiang).

Extraction and identification of Pol α , Pol β , Pol γ and the detection of activity of Pol^[7]

Inhibitory experiment of drugs The method was same with that for detecting the activity of Pol. The IC_{50} were calculated in IBM computer by weighted probit analysis (The Programs of Medical Statistics, POMS-2.00)

Inhibitory mode of drugs on Pol α , β , γ ^[8] Mit 0, 2.4, and 3.6 $\text{mg} \cdot \text{L}^{-1}$ were used.

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The reaction was detected with Act DNA 10, 20, 40, 80, and 100 $\text{mg} \cdot \text{L}^{-1}$. There were 3 parallel tubes at each concentration of Act DNA. The data were treated by double reciprocal. The curves of reaction velocity against concentration of primer-template were plotted, the reciprocal of velocity ($1/V$) as ordinate, the reciprocal of concentration of Act DNA ($1/[\text{Act DNA}]$) as abscissa. The conclusion was made according to the curves and the K_i value was counted^[8].

RESULTS

Identification of Pol α , β , and γ Three peaks of activity (F_1 , F_2 , F_3) were found in the sequence of washing out. According to the relation of activity of enzyme with ionic strength, F_1 , F_2 , and F_3 were identified with Pol α , γ , and β , respectively. F_1 was inhibited by aphidicolin, the selective inhibitor of Pol α , the inhibitory rate was 54.5%, but F_2 and F_3 could not be inhibited by aphidicolin, which was conformable to the literatures.

Effects of Mit and Dox on Pol α , β , and γ Mit 2.5, 5, 10, 20, and 50 $\text{mg} \cdot \text{L}^{-1}$ inhibited DNA Pol α , β , and γ (Fig 1).

IC_{50} values of Mit on DNA Pol α , β , and γ were 11.9, 6.5, and 11.9 $\mu\text{mol} \cdot \text{L}^{-1}$, respectively (Tab 1).

The K_i values of Mit on Pol α , β , and γ were 1.86 ± 0.21 , 2.22 ± 0.08 , and $2.05 \pm 0.46 \mu\text{mol} \cdot \text{L}^{-1}$, respectively. The 3 lines intersected at the same point on the Y-axis (Fig 2).

Tab 1. IC_{50} and 95% confidence ($\mu\text{mol} \cdot \text{L}^{-1}$) of Mit and Dox on Polymerases.

	Mitoxantrone	Doxorubicin
Pol α	11.9 (10.3 - 13.7)	30.9 (22.8 - 41.9)
Pol β	6.5 (4.0 - 10.8)	22.1 (18.8 - 25.9)
Pol γ	11.9 (9.1 - 15.7)	21.7 (18.2 - 25.9)

DISCUSSION

Mit, an antitumor drug, is extensively used in clinical cancer treatment. It was considered that Mit intercalated into DNA and stabilized DNA double helix, leading to DNA single helix or double helix breaking^[6]. In this paper, researches indicated that Mit was also an inhibitor of Pol. The Pol α , β , and γ of Ehrlich ascites tumor cells of mice were strongly inhibited by Mit. Pol δ of Novikoff hepatoma cells was insensitive to Mit, regardless of the primer-template used, quite in contrast to DNA Pol α and Pol ϵ which were strongly inhibited by Mit^[9]. Even though our experimental model and subunit of Pol used were different from those of [9], but the results from the 2 laboratories confirmed the same conclusion: Mit is an inhibitor of DNA polymerase of tumor cells. It is a new complement on the mechanism of antitumor action of Mit.

In our study, it was also indicated that the inhibitory mode of Mit on Pol was competitive, suggesting that the inhibition of Pol α , β , and γ by Mit was competition with respect to template DNA, it is not yet reported in literature.

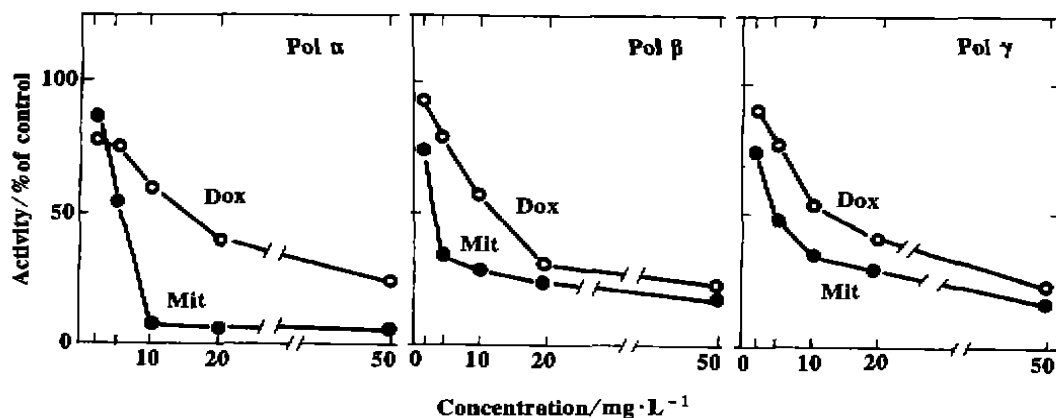


Fig 1. Effects of mitoxantrone (Mit) and doxorubicin (Dox) on polymerase α , β , and γ .

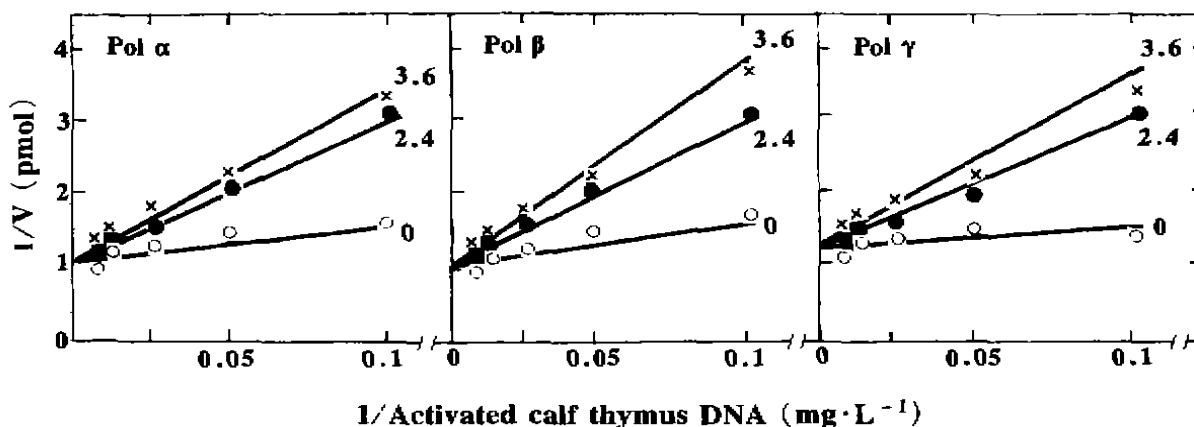


Fig 2. Mode of inhibition of polymerase α , β , and γ by mitoxantrone 0, 2.4, and 3.6 $\text{mg} \cdot \text{L}^{-1}$.

We detected simultaneously the IC_{50} of Dox on Pol, the result was conformed to literatures^[10]. In the same condition of experiment, the IC_{50} of Dox on Pol was higher than that of Mit. It means that the inhibitory potency of Mit on Pol is stronger than that of Dox. Mit was a weaker intercalator of DNA, since the alkylamino chains prevent the chromophore from perfect insertion between base pairs^[1]. Combining our study, it seems that in the antitumor mechanisms of Mit, the inhibitory effect on Pol may be more important than the intercalation of DNA.

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米托蒽醌对艾氏腹水癌细胞 DNA 多聚酶的影响¹

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关键词 米托蒽醌; 酶抑制剂; DNA 多聚酶; Ehrlich 瘤癌; 多柔比星; 阿非迪霉素

目的: 研究米托蒽醌(Mit)对肿瘤细胞 DNA 多聚酶的影响. 方法: 用磷酸纤维柱层析法分离, 纯化艾氏腹水癌细胞 DNA 多聚酶 α , β , γ , 并加以鉴定. 测定 Mit 对多聚酶的影响. 结果: Mit 抑制多聚酶 α , β , γ , IC_{50} 分别为: 11.9, 6.5, 11.9 $\mu\text{mol} \cdot \text{L}^{-1}$. K_i 值分别为: 1.86, 2.22, 2.05 $\mu\text{mol} \cdot \text{L}^{-1}$. Mit 对 DNA 多聚酶 α , β , γ 的抑制方式是竞争性抑制. 结论: Mit 是肿瘤细胞 DNA 多聚酶 α , β , γ 的强力抑制剂, 抑制方式是竞争模板 DNA.

Protective effects of *Ginkgo biloba* extract against lysophosphatidylcholine-induced vascular endothelial cell damage

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KEY WORDS *Ginkgo biloba*; flavones; ginkgolides; lysophosphatidylcholines; vascular endothelium; epoprostenol; malondialdehyde; thoracic aorta; vitamin E

AIM: To study the protective effects of *Ginkgo biloba* extract (*GbE*) against endothelial cell damage induced by lysophosphatidylcholine (LPC). **METHODS:** The vasorelaxation response to acetylcholine (ACh) were investigated in the isolated rabbit thoracic aorta. Lipid peroxidation products were determined by measuring thiobarbituric acid reactive substance.

RESULTS: *GbE* attenuated the inhibition of vasorelaxation response to ACh and prevented the LPC-induced increase of malondialdehyde (MDA) content both in thoracic aortae. *GbE* prevented the leakage of LDH and the increase of MDA content in cultured endothelial cells in a concentration-dependent manner. *GbE* also markedly increased epoprostenol level in cultured endothelial cells treated with LPC.

CONCLUSION: *GbE* protected endothelial cells against LPC-induced damage due to reduction in lipid peroxidation and facilitation of synthesis and/or release of epoprostenol.

In atherosclerotic arteries, endothelium-dependent relaxation was attenuated^[1]. Much evidence showed that injury of endothelial cell was an initial event in the development of atherosclerosis. Oxidized low density lipoprotein (ox-LDL) inhibited endothelium-dependent vasorelaxation of normal arteries and stimulated proliferation of vascular smooth muscle cell^[2]. LDL oxidation was associated with an increase of lysophosphatidylcholine (LPC) content^[3] and ox-LDL-induced inhibition on endothelium-derived relaxing factor (EDRF) might attribute to the increase of LPC, which enhanced vascular

superoxide anion production and impaired the release of EDRF via activation of protein kinase C^[4,5]. Thus LPC was considered as an active principal component of ox-LDL.

Ginkgo biloba extract (*GbE*) has been used in traditional Chinese medicine for centuries. *GbE* is a potent scavenger of several reactive oxygen species such as singlet oxygen, superoxide anions, and hydroxyl radicals^[6,7]. In normal endothelial cells *GbE* stimulated epoprostenol production and released EDRF at a low concentration^[8]. *GbE* protected the heart against ischemia-reperfusion, probably an oxygen free radical-induced damage^[9] and against endothelial dysfunction evoked by oxygen free radicals^[10]. The present paper was to study the protective effects of *GbE* against the LPC-induced damage of endothelial cells.

MATERIALS AND METHODS

Drugs and chemicals *GbE* (standardized to contain 24 % flavonoid glycosides, 6 % ginkgolides, and 70 % of other substances: proanthocyanidins, organic acids, sugars, etc^[11], No 961027) was produced by Shanghai Lityuan Industry Company Ltd. Acetylcholine hydrochloride (ACh), phenylephrine hydrochloride (Phe), and chemicals used in preparing Krebs' solution were products of Shanghai Reagents Co, China. LPC, tocopherol (vitamin E, Vit E), trypsin, and M199 medium were purchased from Sigma Chemical Co, USA. Thiobarbituric acid was a product of Fluka, Switzerland. Lactate dehydrogenase (LDH) kit was obtained from Clinic Reagents Co, Beijing. Thromboxane B₂ (TXB₂) and 6-keto-PGF_{1α} kit were the products of Suzhou Medical College.

Preparation of aortic ring and bioassay of vasoreactivity New Zealand white rabbits of either sex (1.5-2.5 kg) were anesthetized with pentobarbital sodium (60 mg·kg⁻¹ iv). The thoracic aorta was excised with intact endothelium

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and cut into rings (4 mm in length). The aortic rings were mounted between stainless steel hooks in organ bath containing Krebs' solution (37 °C, bubbled with 95 % O₂ + 5 % CO₂ mixture). The ring was connected to force transducers, loaded with 5 g on resting tension, and equilibrated for 60 min. The ring was precontracted by Phe 0.01 μmol · L⁻¹. After the contraction was stabilized, rings were relaxed by ACh 4 μmol · L⁻¹.

The first ACh-induced vasorelaxation was calculated as percentage of the contraction by Phe. After a control experiment to assure the competency of the aortic ring, Phe and ACh were washed out and the ring was equilibrated again, followed by preincubation with LPC (4 mg · L⁻¹) for 30 min. For the studies on the effect of GbE on the inhibition by LPC of vasodilator response to ACh, GbE (10, 25, 50 mg · L⁻¹) were added into the tissue bath 30 min before exposure of ring to LPC. In positive drug control group, Vit E (100 mg · L⁻¹) was dissolved in 10 % ethanol first and added to perfusate before the exposure to LPC. After another 30-min preincubation, the second ACh-induced vasorelaxation was monitored. The "relaxation ratio," a parameter of the reduction in the capacity to release EDRF, was calculated^[12]:

$$\text{Relaxation ratio} = \% \text{ of relaxation after treatment} / \% \text{ of relaxation before treatment}$$

Determination of tissue MDA content

After each bioassay of vasoreactivity, aortic rings were rapidly frozen in liquid nitrogen and preserved at -20 °C until assay. Lipid peroxides were assayed according to the thiobarbituric acid method^[13]. Briefly, the aortic ring was cut into small pieces in iced phosphate buffer solution at a ratio of 20 mL:1 g and homogenized in an ice bath. The homogenate was centrifuged at 2000 × g at 4 °C for 10 min and the supernatant was assayed for MDA level and thiobarbituric acid reactive substance (TBARS) content by a spectrophotometer.

Preparation of pig aortic endothelial cell cultures Pig aortic endothelial cells were harvested and cultured^[14]. Having been excised from pig, the aorta was immediately flushed with sterile D-Hanks' solution containing benzylpenicillin 100 kU · L⁻¹ and streptomycin 100 mg

· L⁻¹. The aorta was ligated at one end and washed with 10 mL D-Hanks' solution. After ligation of the intercostal arteries, trypsin (0.25 %) was infused into the lumen and the aorta was incubated at 37 °C for 30 min. The endothelial cells were collected and washed twice by repeatedly spinning and resuspending the cells in M199 medium. Cells were finally suspended in M199 supplemented with 20 % heat-inactivated fetal bovine serum (FBS), benzylpenicillin 100 kU · L⁻¹ and streptomycin 100 mg · L⁻¹. The cells harvested from each aorta were seeded into a culture flask (20 cm²) and grown in an incubator at 37 °C in 5 % CO₂ in air. The culture medium was replaced with fresh media every 2 d. All culture cells showed typical morphology. Experiments were performed on 3 - 4 passages of endothelial cells.

Experimental procedures of cultured endothelial cells After 3-d serum-free medium culture, endothelial cells were used in 6 groups: 1) Control; 2) LPC 4 mg · L⁻¹; 3) GbE₁ 10 mg · L⁻¹ + LPC; 4) GbE₂ 25 mg · L⁻¹ + LPC; 5) GbE₃ 50 mg · L⁻¹ + LPC, and 6) Vit E (100 mg · L⁻¹) + LPC. GbE and Vit E were added 3 h before exposure to LPC. After incubation with LPC for 30 min, the culture medium and cells were collected and stored at -20 °C for assay of LDH, MDA, TXB₂, and 6-keto-PGF_{1α}. LDH was spectrophotometrically measured using LDH kits. The content of thiobarbituric acid reactive substance, reflecting level of lipid peroxidation, was measured by a spectrophotometer and expressed as the amount of MDA. TXB₂ and 6-keto-PGF_{1α} were assayed by radioimmunoassay.

Statistics The data were expressed as $\bar{x} \pm s$ and analyzed by the Newman-Keuls test.

RESULTS

LPC-attenuated vasorelaxation responses of aortic ring to ACh Relaxation ratio of aortic rings to ACh was markedly reduced after 30-min incubation with LPC 4 mg · L⁻¹. GbE itself had no effect on Phe-induced contraction. However, pretreatment with GbE markedly attenuated the inhibition of vasorelaxation response to ACh by LPC in a concentration-dependent manner. Preincubation with Vit E 100 mg · L⁻¹ markedly prevented the LPC-attenuated vasorelaxation

responses to ACh (Tab 1).

MDA content of aortic rings The MDA content in aortic tissue was increased after the exposure of rings to LPC for 30 min. GbE and Vit E prevented the rise of MDA (Tab 2).

Tab 1. Effect of GbE on inhibition of vasorelaxation response to ACh induced by LPC in rabbit thoracic aorta. $\bar{x} \pm s$. ^c $P < 0.01$ vs control. ^f $P < 0.01$ vs LPC.

Drug/mg·L ⁻¹	Rings	Relaxation ratio
Control	6	0.98 ± 0.20
LPC 4	16	0.35 ± 0.20 ^c
GbE ₁ 10 + LPC 4	12	0.60 ± 0.18 ^f
GbE ₂ 25 + LPC 4	7	0.69 ± 0.18 ^f
GbE ₃ 50 + LPC 4	9	0.70 ± 0.09 ^f
Vit E 100 + LPC 4	5	0.80 ± 0.19 ^f

Tab 2. Effect of GbE on changes in the levels of malondialdehyde (MDA) induced by LPC in rabbit thoracic aorta. $\bar{x} \pm s$. ^c $P < 0.01$ vs control. ^f $P < 0.01$ vs LPC.

Drug/mg·L ⁻¹	Rings	MDA (nmol/g aorta)
Control	6	1.8 ± 0.7
LPC 4	18	7.9 ± 2.4 ^c
GbE ₁ 10 + LPC 4	10	2.8 ± 1.4 ^f
GbE ₂ 25 + LPC 4	9	2.5 ± 0.5 ^f
GbE ₃ 50 + LPC 4	9	3.1 ± 1.3 ^f
Vit E 100 + LPC 4	6	2.0 ± 1.3 ^f

Morphology of endothelial cells Preincubation with LPC in serum-free medium cultured endothelial cells for 30 min caused apparent morphological damage in endothelial cells within a few hours. The endothelial cells exhibited a contraction and irregular arrangement. Pretreated with GbE, they maintained the normal cobble stone pattern after exposure to LPC in a concentration-dependent manner.

LDH release Exposure to LPC for 30 min remarkably increased LDH content in medium. GbE also inhibited the rise of LDH in a concentration-dependent manner. These results indicated that GbE reduced endothelial toxicity induced by LPC. The same protective effect was observed in Vit E group (Tab 3).

MDA level of cultured endothelial cells

Incubation of endothelial cells with LPC increased the MDA level. Preincubation with GbE (10 – 50 mg·L⁻¹) attenuated the LPC-induced rise of

MDA (Tab 4).

TXB₂ and epoprostenol content LPC reduced the content of epoprostenol, GbE (10 – 50 mg·L⁻¹) prevented this reduction. However, the change of TXB₂ was insignificant (Tab 4).

Tab 3. Effect of GbE on lactate dehydrogenase leakage induced by lysophosphatidylcholine (LPC) in the medium. $\bar{x} \pm s$. ^c $P < 0.01$ vs control. ^e $P < 0.05$, ^f $P < 0.01$ vs LPC.

Drug/mg·L ⁻¹	Samples	LDH/IU·L ⁻¹
Control	17	25 ± 9
LPC 4	13	39 ± 7 ^c
GbE ₁ 10 + LPC 4	6	32 ± 3
GbE ₂ 25 + LPC 4	6	28 ± 6 ^e
GbE ₃ 50 + LPC 4	6	27 ± 5 ^f
Vit E 100 + LPC 4	6	23 ± 6 ^f

Tab 4. Effect of GbE on TXB₂, 6-keto-PGF_{1α} and MDA content of pig aortic endothelial cells in presence of LPC. $n = 6$ samples, $\bar{x} \pm s$. ^c $P < 0.01$ vs control. ^e $P < 0.05$; ^f $P < 0.01$ vs LPC.

Drug/mg·L ⁻¹	TXB ₂ /ng·L ⁻¹	6-keto-PGF _{1α} /ng·L ⁻¹	MDA/μmol·L ⁻¹
Control	237 ± 35	64 ± 27	2.4 ± 0.5
LPC 4	271 ± 32	29 ± 9 ^c	13.7 ± 1.8 ^c
GbE ₁ 10 + LPC 4	293 ± 36	49 ± 12 ^e	12.5 ± 1.2
GbE ₂ 25 + LPC 4	309 ± 21	41 ± 10 ^e	7.7 ± 1.1 ^f
GbE ₃ 50 + LPC 4	288 ± 21	51 ± 22 ^e	5.4 ± 1.3 ^f
Vit E 100 + LPC 4	320 ± 41	42 ± 21 ^e	7.0 ± 1.8 ^f

DISCUSSION

GbE protected the heart against ischemia-reperfusion injury and its cardio-protective mechanism was correlated with its ability to prevent lipid peroxidation^[9,15].

Our present experiments suggested that GbE protect the endothelial cells and aortic endothelium from histomorphological and functional changes and against LPC-induced damage by maintaining the release of EDRF in isolated rabbit thoracic aorta, suppressing elevation of MDA and LDH content, and increasing the level of 6-keto-PGF_{1α} in cultured endothelial cells.

GbE is known to scavenge superoxide anions and suppress the lipid peroxidation in both tissue and plasma during ischemia-reperfu-

sion^[15]. Our present data showed that GbE inhibited the increase of MDA content elicited by LPC. These results were in accordance with those observed in myocardial reperfusion injuries^[15]. Therefore, our results together with findings by others suggested that the preventive effect of GbE on endothelial cells might be due to its anti-oxygen free radical and anti-peroxidation.

To explore whether the protective effect of GbE against damages of endothelial cells induced by LPC was associated with its stimulation on epoprostenol formation, we measured the content of 6-keto-PGF_{1α}, a stable metabolite of epoprostenol in cultured endothelial cells incubated with LPC. Our data showed that GbE increased the content of epoprostenol, suggesting that the protective role of GbE on endothelial cells at least be partly mediated by the facilitation of the release of epoprostenol and thus the protective effects of epoprostenol may attribute to its membrane-stabilizing action and possibly to its direct anti-oxygen free radical and anti-lipid peroxidation, similar to an anti-oxidant Vit E.

In conclusion, GbE possessed a protective effect on endothelial cells against injury elicited by LPC, a principal component of ox-LDL, suggesting that GbE may contribute to the prevention and treatment of atherosclerosis. The mechanism of GbE action may be related to its facilitation of the synthesis and/or release of epoprostenol and its antioxidant effect resulted in the reduction in lipid peroxidation.

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 银杏叶提取物保护血管内皮细胞
 免受溶血磷脂酰胆碱损伤 12285.5

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关键词 银杏; 黄酮; 银杏内酯; 溶血磷脂酰胆碱类; 血管内皮; 依前列醇; 丙二醛; 胸主动脉; 维生素 E

目地: 研究银杏叶提取物 (GbE)对氧化低密度脂蛋白主要成分溶血磷脂酰胆碱(LPC)引起内皮细胞损伤的拮抗作用。方法: 离体兔胸主动脉对乙酰胆碱(ACh)血管舒张效应。硫代巴比妥酸法测