ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Sep; 21 (9): 835 – 840
http://www.chinaphar.com

Inhibitory effects of purified green tea epicatechins on contraction and proliferation of arterial smooth muscle cells

CHEN Zhen-Yu, LAW Wai-Ip, YAO Xiao-Qiang, LAU Chi-Wai, HO Water Kwok Keung, HUANG Yu[1]
(*Departments of Biochemistry and Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Shatin, Hong Kong, China*)

KEY WORDS tea; catechols; proliferation; vasodilation; smooth muscle; arteries

ABSTRACT

AIM: The present study was aimed to examine the vasorelaxant and antiproliferative responses to purified green tea epicatechin mixture, (–)epicatechin and (–)epigallocatechin gallate on rat arterial smooth muscle cells. **METHODS**: Changes in isometric force were measured by Grass force transducer and effects on cell proliferation were evaluated using [^{3}H]thymidine incorporation assay. **RESULTS**: Epicatechin mixture, (–)epicatechin and (–)epigallocatechin gallate, which we isolated and purified from jasmine green tea, concentration-dependently, reduced the contractile response to phenylephrine in rat isolated aortic rings with (–)epigallocatechin gallate being more effective. These three agents also inhibited [^{3}H]thymidine incorporation into DNA in cultured rat aortic smooth muscle cells in a concentration-dependent manner. **CONCLUSION**: The purified epicatechin derivatives from jasmine green tea relaxed the isolated rat arteries preconstricted by phenylephrine and inhibited aortic smooth muscle cell proliferation.

INTRODUCTION

Green tea is the most commonly consumed beverage in the oriental region. Epicatechins purified from jasmine green tea have been recently shown to relax the rat blood vessels[1] and endothelial nitric oxide contributes towards the (–)epicatechin-mediated relaxant response in rat mesenteric arteries[2]. Green tea extract decreased the plasma cholesterol and triglyceride levels in the rats[3]. Tea polyphenols are powerful antioxidants[4] that may play a role in lowering the oxidation of low-density lipoproteins[5,6] and induce a gradual regeneration of α-tocopherol[7]. Oxidative modification of low-density lipoprotein plays a significant role in the development of atherosclerosis[8]. Besides, we have recently reported that the hypolipidemic effect of green tea epicatechins is not due to inhibition of biosynthesis of cholesterol or fatty acid but is most likely related to their effects on absorption of dietary fat and cholesterol[9]. Vascular smooth muscle proliferation is an another essential factor contributing to the formation of atherosclerotic plagues[10]. It is possible that drinking green tea may have beneficial effects on the cardiovascular function by inhibition of vascular contraction and vascular smooth muscle cell proliferation. The present study was therefore aimed to examine the possible relaxant and antiproliferative effects of purified jasmine green tea epicatechin derivatives in rat arterial smooth muscle cells.

MATERIALS AND METHODS

Isolation and purification of GTE, EC, and EGCG The method described by Agarwal *et al* (1992)[11] was employed to extract total green tea epicatechin (GTE) from jasmine tea. The extraction method used in our previous work yielded about 7.4 g epicatechins out of 100 g dry tea leaves, and over 99 % purity was achieved[1,12]. Both (–)epicatechin (EC) and (–)epigallocatechin gallate (EGCG) were isolated and purified according to procedures reported before[12]. In the batch of GTE used in this study, EGCG was the major ingredient (62.3 %, w/w) followed by (–)epicatechin gallate (19.2 %), (–)epigallocatechin (8.3 %) and (–)EC (4.6 %). We chose EC and EGCG for the present study because EC was found to be relatively stable in the bloodstream after oral administration[12] and EGCG was the most potent antioxidant among the four derivatives[6].

Tissue preparation After an approval for use of

[1] Correspondence to HUANG Yu, PhD. Phn 285-2609-6787. Fax 285-2603-5022. E-mail yu-huang@cuhk.edu.hk
Received 2000-01-31 Accepted 2000-05-04

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Sep; 21 (9)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

laboratory animals was obtained from Chinese University of Hong Kong, male Sprague-Dawley rats weighing 300 g were sacrificed by cervical dislocation. The thoracic aorta or the superior branch of mesenteric artery was excised and cut into three-four 3 mm-wide ring segments after the adhering connective tissues were carefully removed. Each arterial ring was dispensed between two stainless wire hooks in a 10-mL organ bath. The upper wire was connected to a force-displacement transducer (Grass Instruments Co, USA) and the lower one was fixed at the bottom of the organ bath. The organ bath was filled with Krebs' solution (mmol/L: NaCl 119, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgCl_2$ 1, KH_2PO_4 1.2, *D*-glucose 11). The bath solution was continuously oxygenated with a mixture of 95 % O_2 and 5 % CO_2, and maintained at 37 ℃ (pH ≈ 7.4). The rings were placed under an optimal resting tension of 1.5 g for aortic rings or 0.5 g for mesenteric arteries, which had been determined by length-tension relationship experiments. Changes in isometric tension were measured with force transducers. Twenty minutes after being set up in organ baths, tissues were first contracted with a single concentration of phenylephrine (0.3 μmol/L for aorta and 1 μmol/L for mesenteric artery) and then relaxed by acetylcholine (1 μmol/L) to test their contractility and integrity of the endothelium, after which time they were rinsed with pre-warmed and oxygenated Krebs' solution several times until the basal level of tension was restored. The rings were then allowed to equilibrate further for 60 min. The resting tension was readjusted when necessary.

Force measurement Two consecutive concentration-response curves were constructed at an interval of 45 min for the contractile response to phenylephrine (0.001 – 10 μmol/L) as control. To examine the effects of GTE, EC or EGCG, each endothelium-intact arterial ring was exposed for 30 min to GTE (30 – 300 μmol/L), EC (100 – 500 μmol/L) or EGCG (30 – 300 μmol/L) at desired concentrations before repeating the second concentration-response curve for phenylephrine. The magnitude of tension developed by graded increases in the phenylephrine concentration was expressed as percentages of the maximal contractile response to phenylephrine in the first concentration-response relationship. Since EC was dissolved in dimethyl sulfoxide (Me_2SO), the control concentration-response curves for the phenylephrine-induced contraction were constructed in the presence of 0.2 % Me_2SO.

[^{3}H]thymidine incorporation Rat aortic smooth muscle cells (A7r5, ATCC, Bethesda, MD, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 units penicillin and 1 g streptomycin per L culture medium. The proliferative response of vascular smooth muscle cells was determined by the uptake of [^{3}H]thymidine. Briefly, A7r5 cells (1×10^4 cells/well) were cultured in a 96-well plate. After 24 h, the confluent smooth muscle cells were rendered quiescent by culturing them for 48 h in 0.4 % (v/v) FBS, together with GTE, EC or EGCG added to the culture medium at desired concentrations. Subsequently, cells were stimulated with 2 % FBS and treated with GTE, EC, or EGCG for 3 d, and then rendered quiescent again in 0.4 % FBS for another 24 h, together with each agent. The cells were finally incubated in medium containing 5 % FBS and each agent for 24 h (Tab 1) before addition of [^{3}H]thymidine (37 kB_q/well, Sigma, St Louis, MO, USA). Cells were collected 6 h later by a cell harvester (Cambridge Technology Inc, USA). [^{3}H]Thymidine incorporation into DNA in A7r5 cells was counted in a scintillation counter. The results were expressed as cpm/well and the antiproliferative effects of GTE, EC, or EGCG were expressed as a percentage of Me_2SO (0.1 %) control.

Tab 1. Chronic catechin treatment protocol for A7r5, rat aortic smooth muscle cells.

Drug treatment	Duration	Culture medium DMEM
–	24 h	+10 % FBS
+	48 h	+0.4 % FBS
+	3 d	+2 % FBS
+	24 h	+0.4 % FBS
+	24 h	+5 % FBS

Catechins (GTE, EC, and EGCG) were initially dissolved in Me_2SO at a higher concentration and were subsequently diluted with culture media so as to maintain Me_2SO at 0.1 % (v/v). Significant time was allowed for the drug to be taken inside the cells for the antiproliferative effect.

Drugs Phenylephrine hydrochloride, acetylcholine hydrochloride, [^{3}H]thymidine (Sigma, St Louis, MO, USA). Green tea epicatechin mixture (GTE), (-)epicatechin and (-)epigallocatechin gallate were isolated and purified in our lab[1,4]. All chemicals except for EC in Me_2SO were dissolved in distilled water and further dilu-

tion was made in fresh Krebs' solution in force measurement experiments. GTE, EC, and EGCG were dissolved in Me_2SO in cell proliferation experiments.

Data analysis To study the effect of epicatechins on the phenylephrine-induced contraction, values of pEC_{50} with 95 % confidence limits (the negative log of the phenylephrine molar concentration that caused 50 % of the maximal contraction) and maximum contraction were compared in the absence and presence of each epicatechin derivative. The effects of GTE, EC, or EGCG on rat aortic smooth muscle cell proliferation were expressed as a percentage of the control growth value with vehicle Me_2SO and pIC_{50} with 95 % confidence limits was calculated as the negative log of the drug molar concentration producing a half-maximal inhibition. The results are expressed as $\bar{x} \pm s$. A probability level of less than 0.05 was regarded statistically different.

RESULTS

Vasorelaxant effects of epicatechins In endothelium-intact rat aortic rings, phenylephrine produced a concentration-dependent contraction [pEC_{50} and the maximal increase of arterial tone: 7.07 ± 0.21 (6.66 – 7.48) and 1.3 ± 0.4 (0.5 – 2.1) g, $n = 10$ for the first concentration-response curve; and 7.09 ± 0.19 (6.71 – 7.46) μmol/L and 1.2 ± 0.5 (0.2 – 2.2) g, $n = 10$ for the second concentration-response curve]. Fig 1A shows that preincubation of aortic rings with GTE (100 – 300 μmol/L) caused a rightward shift of the concentration-response curve for the phenylephrine-induced contraction with decreased maximum response (Tab 2). GTE (30 – 300 μmol/L) also concentration-dependently reduced the phenylephrine-induced contractile response in isolated rat mesenteric arteries (Fig 1B, Tab 2). Pretreatment of rings with EC (100 – 500 μmol/L) or EGCG (30 – 300 μmol/L) inhibited the contraction induced by phenylephrine and EGCG was much more effective than EC (Fig 2, Tab 2). The pEC_{50} values and the maximum contraction induced by phenylephrine under various treatments are summarized in Tab 2. It is evident that GTE, EC, or EGCG all suppressed the contractile response to phenylephrine. While GTE (300 μmol/L), EC (500 μmol/L) or EGCG (300 μmol/L) had no effect on the basal tone ($n = 5$ in each case).

Inhibitory effects of epicatechins on [^{3}H]thymidine incorporation into DNA in aortic smooth muscle cells In cultured A7r5 rat aortic smooth muscle cells, GTE (1 – 500 μmol/L) inhibited [^{3}H]thymidine incorporation into DNA in a concentration-dependent manner with pIC_{50} value of 5.48 ± 1.12 (3.28 – 7.67) ($n = 6$) and 98.5 % ± 0.3 % maximum inhibition (Fig 3). Similarly, EC (0.1 – 500 μmol/L) or EGCT (0.1 – 300 μmol/L) also concentration -dependently suppressed the aortic smooth muscle cell proliferation except for the concentration of 0.3 μmol/L that caused an increase in cell proliferation (Fig 4). The pIC_{50} values were 4.76 ± 0.71 (3.37 – 6.15) for EC ($n = 12$, Fig 4A) and 5.56 ± 0.38 (4.81 – 6.30) for EGCG ($n = 12$, Fig 4B). The control value for [^{3}H]thymidine incorporation was presented as a percentage of control in the presence of vehicle (0.1 % Me_2SO) was 38 686 ± 3 061 (32 686 – 44 685) cpm per well (1×10^4 cells) ($n = 6$). GTC 30 μmol/L, EC 100 μmol/L or EGCG 30 μmol/L almost totally suppressed cell proliferation (Fig 3, 4).

Tab 2. pEC_{50} values and maximal response for the phenylephrine-induced contraction in rat arteries.

Treatment	pEC_{50}	E_{max}/%	n
GTE in aorta/μmol·L^{-1}			
0	7.09 ± 0.19	98 ± 8	10
100	6.47 ± 0.35^b	46 ± 25	5
300	6.46 ± 0.35^b	17 ± 5^b	5
EC in aorta/μmol·L^{-1}			
0	7.06 ± 0.19	98 ± 10	10
100	6.76 ± 0.24	101 ± 22	5
300	6.71 ± 0.25^b	80 ± 18	5
500	6.75 ± 0.25^b	86 ± 7^b	5
GTE in mesenteric artery/μmol·L^{-1}			
0	5.93 ± 0.36	99 ± 2	8
30	5.95 ± 0.30	93 ± 16	5
100	5.43 ± 0.09^b	61 ± 27	5
300	5.61 ± 0.06^b	21 ± 10^b	5
EGCG in aorta/μmol·L^{-1}			
0	7.08 ± 0.19	98 ± 9	10
30	6.68 ± 0.39^b	80 ± 24	5
100	6.31 ± 0.17^b	67 ± 18	5
300	6.61 ± 0.31^b	35 ± 10^b	5

EC_{50} and the maximum contraction (E_{max}) were obtained in the second concentration-response curves for the contractile response to phenylephrine. Drug at each concentration was incubated for 30 min before repeating the second concentration-response curve. Data are $\bar{x} \pm s$. $^bP < 0.05$ *vs* control group (0.2 % Me_2SO in EC group and Krebs'solution in other groups). GTE, epicatechin mixture. (EC, EGCG).

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Sep; 21 (9)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

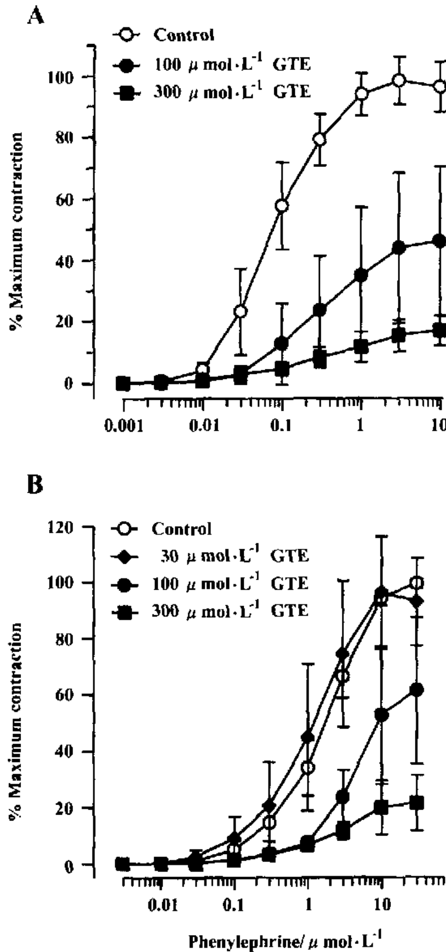

Fig 1. Effect of GTE on the contractile responses of rat aortic rings (A) and mesenteric arteries (B) to phenylephrine in the absence (○, n = 8 – 10) and presence of green tea epicatechin mixture (GTE) (◆, 30 μmol/L, n = 5; ●, 100 μmol/L, n = 5; ■, 300 μmol/L, n = 5). Tissues were exposed to each agent for 30 min before repeating the second concentration-response curve. Curves were drawn by joining the adjacent data points. Values are $\bar{x} \pm s$ from n experiments.

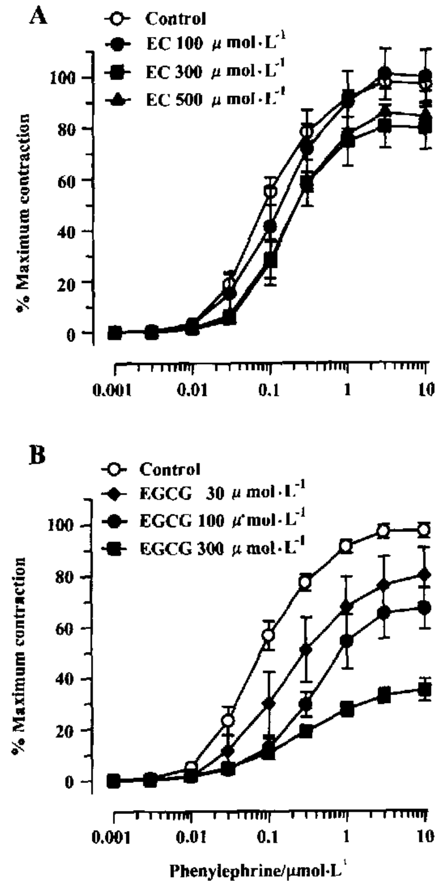

Fig 2. (A) Logarithmic concentration-response curves of contractile responses of rat aorta for phenylephrine in the absence (○, n = 10) and presence of EC (●, 100 μmol/L, n = 5; ■, 300 μmol/L, n = 5; ▲, 500 μmol/L, n = 5). (B) Logarithmic concentration-response curves of contractile responses of rat aorta for phenylephrine in the absence (○, n = 10) and presence of EGCG (◆, 30 μmol/L, n = 5; ●, 100 μmol/L, n = 5; ■, 300 μmol/L, n = 5). Tissues were exposed to each agent for 30 min before repeating the second concentration-response curve. Values are $\bar{x} \pm s$ from n experiments.

DISCUSSION

The present results show that purified green tea epicatechin mixture or individual derivatives such as EC and EGCG, relaxed the phenylephrine-preconstricted rat aorta or mesenteric arteries with functional endothelium in a concentration-dependent fashion. The observed vasorelaxant effects are unlikely due to the possible antagonistic effects of epicatechins on α_1-adrenoceptor on arterial smooth muscle cells since we have reported earlier that EC also reduced the contractile response to endothelin 1[1] and to the prostaglandin receptor agonist U46619[2]. We have recently demonstrated that EC could act on both endothelium and the underlying vascular smooth muscle cells. The endothelium-derived nitric-oxide/cyclic GMP-mediated mechanism contributed in part to the EC-induced relaxation in rat mesenteric arteries[2]. Besides,

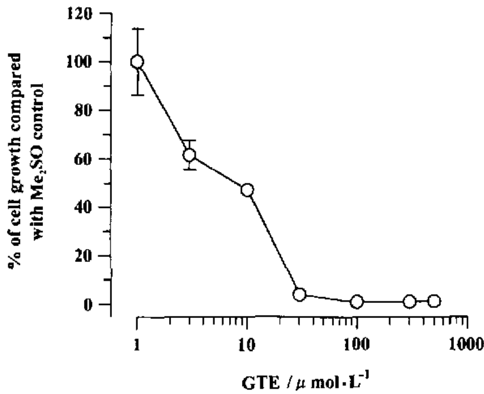

Fig 3. Inhibitory effect of green tea epicatechin mixture on [^{3}H] thymidine incorporation into rat aortic smooth muscle cells (A7r5). The results are $\bar{x} \pm s$ of 12 experiments.

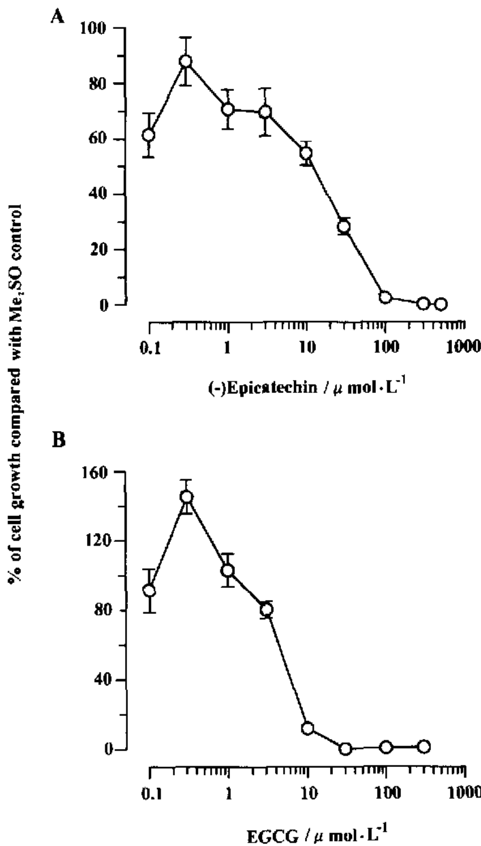

Fig 4. Inhibitory effect of EC (A) and EGCG (B) on [^{3}H] thymidine incorporation into rat aortic smooth muscle cells (A7r5). The results are $\bar{x} \pm s$ of 6 experiments.

EC-induced relaxation was attenuated by the large-conductance Ca^{2+}-activated K^+ channel blocker iberiotoxin only in endothelium-intact arteries but not in rings pretreated with N^G-nitro-*L*-arginine methyl ester, an inhibitor of endothelial nitric-oxide synthase[2], thus further indicating a significant role of the endothelium.

It is generally believed that α_1-adrenoceptor agonist-induced steady tension is caused by increased intracellular Ca^{2+} concentration and that protein kinase C is involved in promoting Ca^{2+} entry via plasma membrane Ca^{2+} channels[13]. However, four epicatechin derivatives did not affect the contractile response to active phorbol ester in mesenteric arteries[1] suggesting that inhibition of the protein kinase C-mediated contractile mechanism may play a little role.

Many factors may contribute towards a long-term benefit of green tea consumption on the cardiovascular system. For instance, arterial smooth muscle proliferation is an important factor involved in the formation of atherosclerotic plagues[10]. In this study, we tested the possible inhibitory effect of epicatechin mixture, EC, and EGCG on proliferation of cultured A7r5 rat aortic smooth muscle cells. We found that GTE, EC, and EGCG significantly suppressed the FBS-stimulated cell proliferation at concentrations smaller than those used to relax the phenylephrine-preconstricted rat arteries and that complete inhibition of cell proliferation could be achieved in the presence of epicatechin derivatives. The difference in effectiveness between the two pharmacological actions is probably caused by a much longer drug exposure time (over a week) in the cell proliferation experiments. Since nitric oxide and nitric oxide donors are reported to exhibit antiproliferative effects on vascular smooth muscle cells[14], it is possible that nitric oxide may also be involved in the anti-atherosclerotic effect of green tea catechins *in vivo*. Oxidative modification of low-density lipoproteins plays a role in the development of atherosclerosis and α-tocopherol serves as a major antioxidant in human low-density lipoproteins. We have currently demonstrated regeneration of α-tocopherol in human low-density lipoprotein by green tea catechins[7] and thus elucidated an additional mechanism to the anti-atherosclerotic effect of drinking green tea. The exact cellular mechanisms underlying the antiproliferative effects of epicatechins remain to be investigated.

The results of the present study show that epicatechin mixture and EGCG display the similar effectiveness in relaxing the blood vessels and inhibiting smooth muscle cell

proliferation while EC is much less potent. EGCG and EC account for 62.3 % and 4.6 %, respectively, of the total amount of purified green tea epicatechins. It is therefore possible that EGCG is the principal active component in the tea catechin mixture that causes both relaxation of rat isolated arteries and inhibition of cell proliferation in our study.

Taken together, our data show that EGCG is the major contributor in purified green tea epicatechin mixture that possesses both vasorelaxant and antiproliferative effects *in vitro* and the concentration of EGCG or EC used for the antiproliferative effect is much lower than that used to relax the rat arteries. It is yet to be determined whether epicatechin mixture or EGCG could lower blood pressure significantly *in vivo*. It is apparent that multiple factors work together to exert the long-term anti-atherosclerotic effect of consumption of green tea.

ACKNOWLEDGMENTS We wish to thank Hong Kong Research Grant Committee for the support of this study.

REFERENCES

1 Huang Y, Zhang AQ, Lau CW, Chen ZY. Vasorelaxant effects of purified green tea epicatechin derivatives in rat mesenteric artery. Life Sci 1998; 63: 275 – 83.

2 Huang Y, Chan NWK, Lau CW, Yao XQ, Chan FL, Chen ZY. Involvement of endothelium/nitric oxide in vasorelaxation induced by purified green tea (–)epicatechin. Biochim Biophys Acta 1999; 1427: 322 – 8.

3 Muramatsu K, Fukuyo M, Hara Y. Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. J Nutr Sci Vitaminol 1986; 32: 613 – 22.

4 Chen ZY, Chan PT. Antioxidative activity of green tea catechins in canola oil. Chem Phys Lipids 1996; 82: 163 – 72.

5 Ding ZY, Chen Y, Zhou M, Fang YZ. Inhibitory effect of green tea polyphenol and morin on the oxidative modification of low-density lipoprotein. Chin J Pharmacol Toxicol 1992; 6: 263 – 6.

6 Zhang A, Chan PT, Luk YS, Ho WKK, Chen ZY. Inhibitory effect of jasmine green tea epicatechin isomers on LDL-oxidation. J Nutr Biochem 1997; 8: 334 – 40.

7 Zhu QY, Huang Y, Tsang D, Chen ZY. Regeneration of α-tocopherol in human low-density lipoprotein by green tea catechin. J Agric Food Chem 1999; 47: 2020 – 5.

8 Jialal I, Devaraj S. Low-density lipoprotein oxidation, antioxidant, and atherosclerosis: a clinical biochemistry perspective. Clin Chem 1996; 4: 498 – 506.

9 Chan PT, Fong WP, Cheung YL, Huang Y, Ho WKK, Chen ZY. Jasmine green tea epicatechins are hypolipidemic in hamsters (*Mesocricetus auratus*) fed a high fat diet. J Nutr 1999; 129: 1094 – 101.

10 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362: 801 – 10.

11 Agarwal R, Katiyar SK, Zaidi SIA, Mukbtar H. Inhibition of skin tumor promoter-caused induction of epidermal ornithine decarboxylase in SENCAR mice by polyphenolic fraction isolated from green tea and its individual epicatechin derivatives. Cancer Res 1992; 52: 3582 – 8.

12 Zhang AQ, Zhu QY, Luk YS, Ho KY, Fung KP, Chen ZY. Inhibitory effects of jasmine green tea epicatechin isomers on free radical-induced lysis of red blood cells. Life Sci 1997; 61: 383 – 94.

13 Shearman MS, Sekiguchi K, Nishizuka Y. Modulation of ion channel activity: a key function of the protein kinase C enzyme family. Pharmacol Rev 1989; 41: 211 – 37.

14 Nakaki T, Nakayama M, Kato R. Inhibition by nitric oxide and nitric oxide-producing vasodilators of DNA synthesis in vascular smooth muscle cells. Eur J Pharmacol 1990; 189: 347 – 53.

绿茶儿茶酚抑制血管收缩及动脉平滑肌细胞增生

陈振宇, 罗伟业, 姚晓强, 刘志伟, 何国强, 黄 聿[1] (香港中文大学医学院生物化学及生理系, 沙田, 香港, 中国)

关键词 茶; 儿茶酚类; 增生; 血管舒张; 平滑肌; 动脉

目的: 本文研究从绿茶分离提纯儿茶酚衍生物的血管舒张和抗平滑肌细胞增生的作用. **方法**: 测定分离的大白鼠动脉及肠系膜动脉的收缩力以及动脉平滑肌增生能力. **结果**: 儿茶酚混合物以及其中两个衍生物(表儿茶酚和没食子表食子儿茶酚)浓度依赖性地舒张去甲肾上腺素收缩的动脉以及抑制血管平滑肌细胞增生. **结论**: 绿茶分离提纯儿茶酚混合物及其中两个衍生物具有血管舒张的功能. 而它们抗血管平滑肌细胞增生的作用更为显著. 没食子表食子儿茶酚是绿茶儿茶酚混合物的主要成份, 它的药理作用明显强过表儿茶酚.

(责任编辑 吕 静)