INDUCTION OF VASORELAXATION IN ISOLATED RABBIT AORTA BY *EPIMEDIUM BREVICORNUM* MAXIM LEAF EXTRACTS

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(Received 13th June 2006, accepted 25th July 2006)

The vasorelaxant effect of *Epimedium brevicornum* Maxim (Berberidaceae) (EB) was studied in isolated rabbit thoracic aorta. Results showed that water extract of EB (EB-W) concentration-dependently antagonized phenylephrine (PE)-induced vasoconstriction at the concentrations ranging from 0.001 to 1 mg/mL with an EC_{50} of 12.4 ± 1.5 μg/mL. EB-W evoked vasorelaxation was significantly, however, not completely inhibited by endothelium removal, N^G-nitro-L-arginine methyl ester (a nitric oxide synthase inhibitor) and 1-*H*-[1,2,4] oxadiazolo [4, 3-α] quinoxalin-1-one (a guanylate cyclase inhibitor) pretreatment, indicated that this vasorelaxant effect to be mediated partially through endothelium-dependent mechanism. The residue relaxation caused by EB-W in PE-precontracted endothelium-denuded preparations was abolished in the presence of high extracellular K^+ concentration (60 mM) and by an ATP-sensitive K^+ channel blocker glibenclamide pretreatment. Taken together, these results suggested that *E. brevicornum* may relax vascular beds via both direct NO releasing effect from endothelium and activation of an ATP- sensitive K^+ channel on smooth muscle.

Key words: *Epimedium brevicornum* maxim, Nitric oxide, Potassium channel, Rabbit thoracic aorta, Vasorelaxation.

INTRODUCTION

Epimedium brevicornum Maxim (Berberidaceae) (EB), an ornamental herb grown in Asia and the Mediterranean region has been used for various medicinal purposes. This plant has proven to have efficacy against cardiovascular diseases and much chronic illness, such as infertility, impotence and senile functional diseases[1]. Furthermore, members of this genus are effective in dilation of vascular beds, decreasing blood pressure and blood fat, inhibition of the platelet aggregation, delaying the formation of thrombi, improving humoral

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and cellular immunity, increasing synthesis of DNA and anti-aging[2]. However, there is no direct evidence to clarify its pharmacological profiles of vasorelaxation until now.

Vascular endothelium plays a pivotal role in the regulation and maintenance of overall cardiovascular homeostasis, achieved via the production of a wide array of biochemical mediators[3,4]. Endothelial dysfunction is common in several chronic disease conditions, such as hypertension, diabetes, and atherosclerosis, as evident by a failure of the blood vessel to mediate acetylcholine (ACh)-induced vasorelaxation[5]. It is evident that the agent that promotes the production of vasorelaxant compounds — nitric oxide (NO), prostanoids, endothelium-dependent hyperpolarizing factor (EDHF), and/or inhibits vasoconstrictors (e.g. free radicals, prostaglandin endoperoxides) may result in better vascular health[6]. Additionally, it has become increasingly apparent that K^+ channel openers appear to hold promise as direct effectors of vascular smooth muscle relaxation[7,8]. In the present study, the association of NO/K^+ pathway and the vasorelaxation effect of *Epimedium brevicornum* were studied.

MATERIALS AND METHODS

1. Chemicals

Acetylcholine (ACh), indomethacin (INDO), N^G-nitro-L-arginine methyl ester (L-NAME), 1-*H*-[1,2,4] oxadiazolo [4,3-α] quinoxalin-1-one (ODQ), phenylephrine (PE), sodium nitroprusside (SNP) and tetraethylammonium (TEA) were purchased from Sigma Chemical Co. (USA). The other reagents were of analytical purity.

2. Preparation of Plant Extracts

The leaves of *E. brevicornum* were purchased from a Chinese herbal drug store in Taipei city, and were authenticated by Professor Chien-Chih Chen, National Research Institute of Chinese Medicine (Taipei, Taiwan). A voucher specimen of this herbal drug was deposited in the National Research Institute of Chinese Medicine, Taiwan, R. O. C. Powdered leaves of *E. brevicornum* (EB) (1 kg) was extracted with distilled water (5 L) at 80 °C for 2 hours to obtain water extract (EB-W) and then was filtered and lyophilized. The extract was dissolved in distilled water to reach a stock concentration of 200 mg/mL and diluted in Krebs solution.

3. Preparation of Rabbit Aorta

This study was approved by the Animal Care Committee of National Research Institute of Chinese Medicine and all efforts were made to minimize animal suffering and to reduce the number of animals. The thoracic aorta was excised from adult (4-5 month) male New Zealand White rabbits (3.0-3.5 kg) under anesthesia with sodium pentobarbital and immediately placed in Krebs solution (mM: NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose 10; $CaCl_2$ 2.5, pH 7.4). The aorta were removed free of connective tissue and

fat, and then cut into rings of approximately 5 mm width. All dissecting procedures were carried out with extreme care to protect the endothelium from inadvertent damage. In some aortic rings, the endothelial layer was mechanically removed by gently rubbing the luminal surface of the aortic ring back and forth several times with plastic tubing. Endothelial integrity or functional removal was verified by the presence or absence, respectively, of the relaxant response to 3 μM ACh[9].

4. Record of Isometric Vascular Tone

The aortic rings were suspended in a tissue bath containing Krebs solution (pH 7.4) at 37 °C, while being continuously bubbled with 95%O_2-5%CO_2. The baseline load placed on the aortic rings was 2.0 g, and the changes in isometric tension were recorded using a force-displacement transducer (Grass FT 03, Quincy, MA, USA) connected to a Grass polygraph recording system (Model 7E). Firstly, the aortic rings were contracted with PE to obtain maximal response then washed every 20 min with Krebs solution until the tension returned to the basal level. Subsequently, the vascular rings were exposed to PE to induce appropriate constriction (about 80% of maximal response) for 30 min, and then aortic relaxation was examined by cumulative addition of various extracts of *E. brevicornum* Maxim (0.001-1 mg/mL). After each test, the aortic rings were washed three times with fresh Krebs solution and allowed for 30 min to equilibrate. The concentration-response curves were determined in the absence and in the presence of various inhibitors and after removal of endothelium. Another series of experiments were designed to study the possible involvement of K^+ channel activation. In brief, the aortic ring was bathed in Krebs solution containing 60 mM KCl throughout the experiment. After 20 min incubation, the tissue was precontracted by PE and the relaxant activity of *E. brevicornum* Maxim was studied and compared with those observed in normal condition.

5. HPLC Analysis of EB-W

EB-W was dissolved in methanol, sonicated for 20 min and then adjusted to a concentration of 0.03 g/mL with methanol. The sample was filtered before applying to high-performance liquid chromatography (HPLC) using Cosmosil 5C_{18}-AR-II (5 μm, 4.6 × 250 mm) column and separated by linear gradient elution using water and acetonitrite (ACN) according to the following profile: 0-20 min, 85% H_2O, 15% ACN; 20-30 min, 70% H_2O, 30% ACN; 30-40 min, 73% H_2O, 27% ACN; 40-50 min, 50% H_2O, 50% ACN; 50-60 min, 100% ACN. The content of icariin (retention time: 36.628 min), an index component existed in *E. brevicornum* was analyzed and naphthalene has been chosen as an internal standard (I.S.)[10].

6. Statistical Analysis

Relaxant responses are expressed as percentage relaxation from PE-precontraction levels running from 0%

to 100% and used in the construction of the concentration-response curves. The EC_{50} (the concentration required to cause half-maximal relaxation) was determined by liner interpolation for each concentration-response curve. Results were expressed as means ± s.e.m. Comparisons were made by Student's *t*-test, *p*-values less than 0.05 were considered significant.

RESULTS

1. Vasorelaxant Effects of EB-W in PE-precontracted Aortic Rings

In tissue pre-contracted with phenylephrine (PE, 0.3 μM), EB-W (0.001 to 1 mg/mL) showed a concentration-dependent vasorelaxat response in the isolated rabbit aorta with an EC_{50} of 12.4 ± 1.5 μg/mL (Fig. 1A). To evaluate endothelium-dependency, aortic rings denuded of endothelium was contracted with PE (0.1 μM) and then exposed to EB-W. The amplitude of the contractile response in endothelium-denuded preparations by 0.1 μM PE was not significantly different from endothelium-intake aorta contracted by 0.3 μM of PE. As shown in Fig. 1A, removal of endothelium significantly but not completely suppress EB-W-induced response from 97.4 ± 3.5 to 61.2 ± 3.7%. Contrarily, acetylcholine (3 μM) induced endothelium-dependent relaxation was abolished by endothelial denudation (91.9 ± 4.8 vs. 0%).

2. Vasorelaxant Effect of EB-W in the Presence of Various Inhibitors

Since EB-W induced a partially endothelium-dependent relaxation in rabbit isolated aortic rings, an attempt was made to investigate which endothelium-derived vasorelaxant factors contributed to. Pretreatment of the tissues with a nitric oxide synthase inhibitor L-NAME (0.1 mM) for 20 min fully blocked acetylcholine (3 μM)-induced relaxation (inhibitory percentage > 97%). The same concentration of L-NAME exhibited a moderate attenuation on EB-W-induced relaxation when compared with control (Fig. 1B). The addition of ODQ (30 μM, a guanylyl cyclase inhibitor) for 30 min almost completely suppressed sodium nitroprusside (SNP, 0.3 μM)-induced relaxation from 83.9 ± 5.7 to 2.2 ± 1.9%, however, slightly attenuated EB-W-induced relaxation to a similar degree as L-NAME pretreatment. On the contrary, indomethacin (30 μM, a cyclooxygenase inhibitor) failed to affect EB-W evoked vasorelaxation.

3. Effects of High K^+ Condition and K^+ Channel Blockers on EB-W-induced Vasorelaxation in Endothelium-denuded Aorta

Endothelium disruption did not abolish the response to EB-W, suggesting the involvement of an endothelium-independent pathway. The following experiments were conducted to evaluate the endothelium-resistant response in endothelium deprived aortic rings. In order to ascertain the contribution of membrane K^+

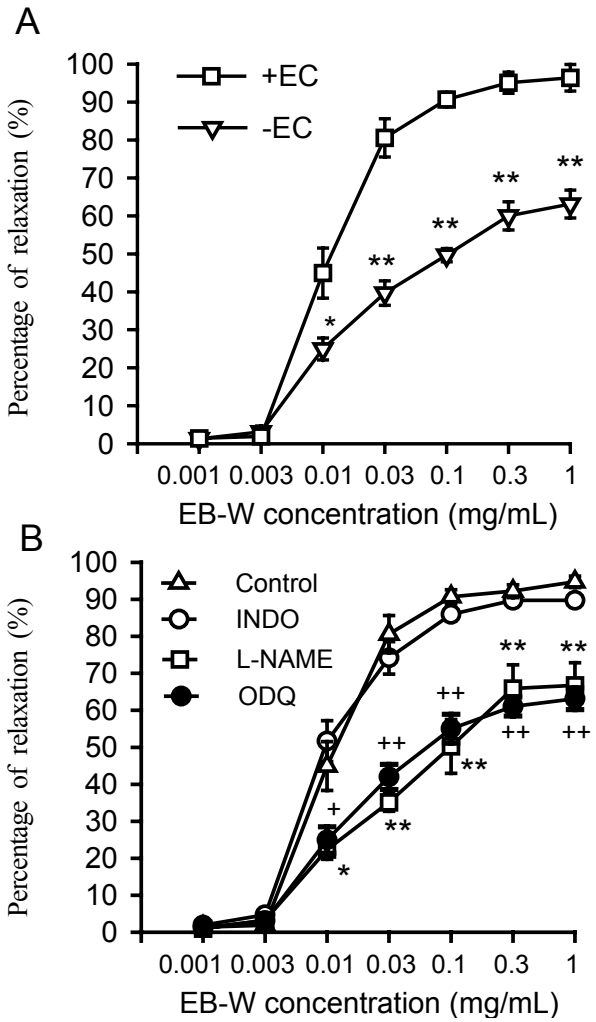

Fig. 1. (A) Relaxation effect of the water extract of *Epimedium brevicornum* Maxim (EB-W) on phenylephrine-precontracted aortic rings with endothelium (+EC) and without endothelium (-EC), respectively. (B) Effect of L-NAME (0.1 mM), ODQ (30 μM) and INDO (30 μM) on EB-W-induced vasorelaxation on phenylephrine-precontracted aortic rings with endothelium. Data represent the mean ± s.e.m. of six individual experiments (n = 8-11). * and + $p < 0.05$, ** and ++ $p < 0.01$, compared with endothelium-intact control. L-NAME, N^G-nitro-L-arginine methyl ester; ODQ, 1-*H*-[1,2,4]oxa-diazolo[4,3-α]quinoxalin-1-one; INDO: indomethacin.

channels to EB-W evoked relaxation, relaxation was conducted in the presence of high extracellular concentration of K^+ (60 mM). Results showed that the relaxant response of EB-W in PE-precontracted rings was disappeared in high K^+ condition (Fig. 2A). To clarify the type of potassium channels, we tested the effect of glibenclamide (an ATP-sensitive K^+ channel blocker) on EB-W evoked relaxation in endothelium-denuded aortic rings. Results showed that, EB-W-induced relaxation was abolished in the presence of glibenclamide (10 μM) (Fig. 2B). The attenuation by glibenclamide resembled that of in high K^+ condition.

4. Icariin Content in Each Extract

Icariin content in EB-W was identified by comparing the retention times (37.0 min) with authentic standards.

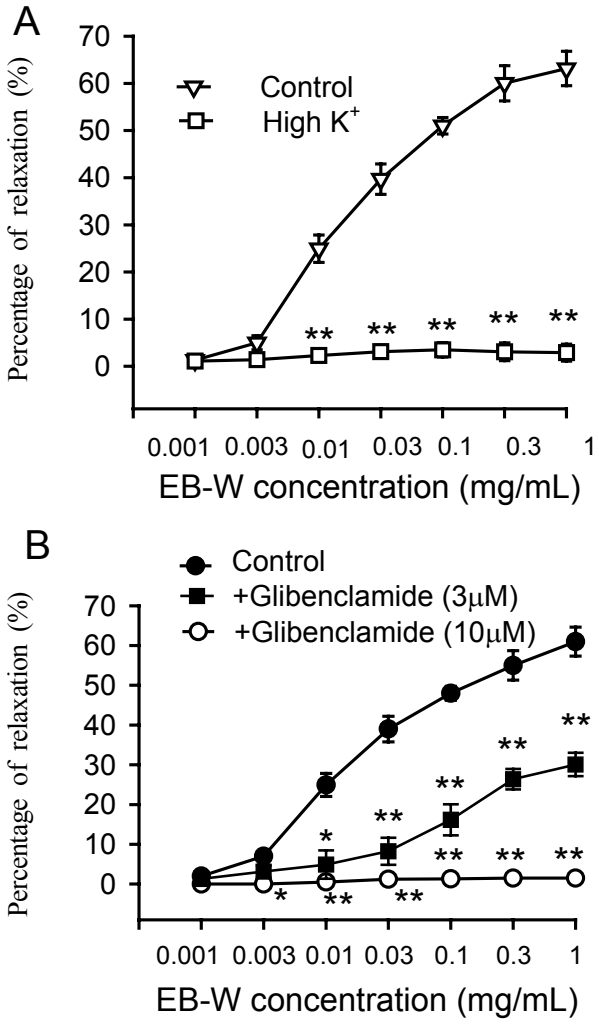

Fig. 2. (A) Comparison of the vasorelaxant effect of the water extract of *Epimedium brevicornum* Maxim (EB-W) under normal and high K^+ (60 mM) conditions in phenylephrine (PE)-precontracted endothelium-denuded aorta. (B) Effect of glibenclamide on EB-W-induced vasorelaxation in PE-precontracted endothelium-denuded aorta. Data represent the mean ± s.e.m. of five individual experiments (n = 6-8). * $p < 0.05$ and ** $p < 0.01$, compared with PE control.

Fig. 3 showed the HPLC profile of EB-W. Analysis showed that EB-W contained 1.17 % of icariin. Thus the vasorelaxant effect of icariin was evaluated in PE-precontracted aortic rings. Our results showed that icariin, even at concentration up to 10 μg/mL, did not show any detectable vasorelaxant effect.

DISCUSSION

E. brevicornum has been reported to have efficacy against cardiovascular diseases such as hypertension. The present study also provided evidence for its potential activity against vasoconstriction. In this study, a conspicuous vasorelaxation was induced by the EB-W in rabbit aorta. These results also revealed that EB-W evoked relaxation in endothelium-intact preparations was significantly reduced, although did not abolish, after endothelial removal and in the presence of a nitric oxide (NO) synthase inhibitor L-NAME. Relaxation of

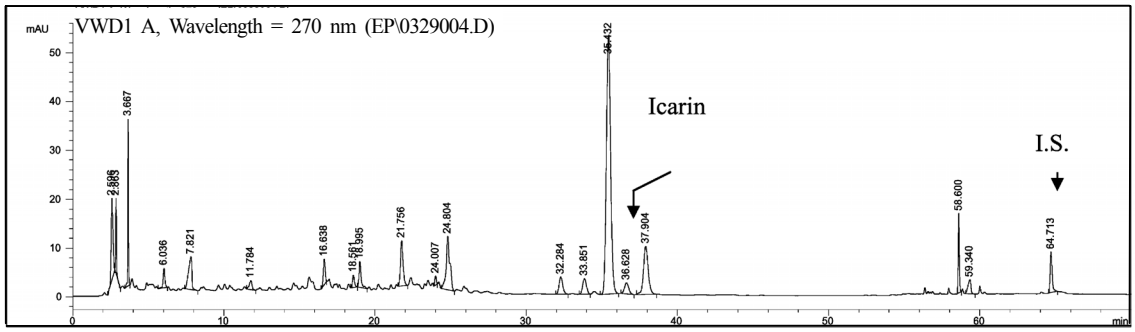

Fig. 3. HPLC chromatogram of the water extract form Epimedium brevicornum Maxim (EB-W). Internal control (naphthalene) was represented as I.S.

smooth muscle by NO involves a sequence of steps. NO formed in the endothelium by the activation of NO synthase, diffuses out of the endothelium to vascular smooth muscle where it binds and activates soluble guanylyl cyclase (sGC). This enzyme catalyzes the conversion of GTP to cGMP and results in relaxation of the smooth muscle[11,12]. The present study also showed that ODQ (an inhibitor of sGC) significantly reduced EB-W-induced relaxation and its influence just resembled to those of removal of endothelium and pretreatment of L-NAME. These results implied that the relaxation of EB-W in endothelium-intact aorta entirely involved the NO-cGMP mediated pathway.

Since endothelium disruption did not abolish the response to EB-W, suggesting an endothelium-independent pathway being involved. It has become increasingly apparent that K^+ channel plays a significant role in maintaining vascular tone[13-15]. Thus, the possibility of K^+ channel activation by EB-W was further investigated. The first evidence of the importance of K^+ channel-mediated hyperpolarization in the action of EB-W was provided by the differential potency of EB-W in normal versus high KCl condition. Vasodilators dependent on the K^+ channel mechanism lose their effects when exposed to high K^+ condition because an increase in extracellular K^+ concentration attenuates the K^+ gradient across the plasma membrane, thus rendering the K^+ channel-activating mechanism ineffective[7,16]. EB-W is suggested to produce relaxation in endothelial denuded preparations mostly in this way since its effect was abolished in the presence of 60 mM KCl. Even a 10-fold increase in the EB-W concentration (10 mg/mL) could not restore significant relaxations (data not shown). Furthermore, EB-W evoked relaxation was counteracted by glibenclamide, an ATP-selective K^+ channel blocker[17]. These data collectively identify K^+ channel as the possible mechanism involved in the direct effect exerted by EB-W on aortic smooth muscle.

Icariin is a major component of flavonoids isolated from *Epimedium brevicornum* Maxim with a wide range of pharmacological and biological activities, including regulating cardiovascular, circulatory, genital and bone marrow systems activity[1,18]. After HPLC analysis and quantitation, the content of icarrin in EB-W was estimated as 1.17 μg/mg. Nevertheless, the present results revealed that icariin, even at concentration up to 10 μg/mL, did not show any detectable vasorelaxant effect in aortic tissues. Since, EB-W is a mixture the effect of

EB-W may be caused either by a single active ingredient or by the combined action of many active agents existed in this extract. Although the active components of EB-W are not yet clear, its physiological effects on vascular beds were correlated with the putative pharmacological activities of *Epimedium brevicornum* which implicate it as a potential for the treatment of hypertension. However, extensive *in vivo* studies and further identification and separation are needed before clinical use. From these results, it can be concluded that EB-W induces both endothelium-dependent and -independent relaxation in rabbit thoracic aorta. Nitric oxide and cGMP are likely to be involved in the endothelium-dependent relaxation, whereas activation of K^+ channels contributed to the endothelium-independent relaxation.

ACKNOWLEDGEMENT

This work was supported by National Research Institute of Chinese Medicine, Taipei, Taiwan, R.O.C.

REFERENCES

1. Wu H, Lien EJ, Lien LL. Chemical and pharmacological investigations of *Epimedium* species: a survey. Prog. Drug Res. 60: 1-57, 2003.
2. Zheng HZ, Dong ZH, She Q. Herba Epimedii. In: Zheng HZ, Dong ZH, She Q. (ed.) Modern Research and Application of Traditional Chinese Medicine. Xueyan press, Beijing, pp. 4225-4254, 1998.
3. Becker, BF, Heindl, B, Kupatt, C, Zahler, S. Endothelial function and homeostasis. Zeitschrift für Kardiologie 89: 160-167, 2000.
4. Rubanyi, GM. The role of endothelium in cardiovascular homeostasis and diseases. J. Cardiovas. Pharmacol. 22: S1-S4, 1993.
5. Brown AA, Hu FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am. J. Clin. Nutri. 73: 673-686, 2001.
6. Radomski MW, Palmer RMJ, Moncada S. Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. Br. J. Pharmacol. 92: 181-187, 1987.
7. Fedida D, Hesketh JC. Gating of voltage-dependent potassium channels. Prog. Biophys. Mol. Bio. 75: 165-199, 2001.
8. Dora KA, Garland CJ. Properties of smooth muscle hyperpolarization and relaxation of K^+ in the rat isolated mesenteric artery. Am. J. Physiol. 280: H2424-H2429, 2001.
9. Chiou WF, Liao JF, Shum AY, Chen CF. Mechanisms of vasorelaxant effect of dehydroevodiamine: a bioactive isoquinazolinocarboline alkaloid of plant origin. J. Cardiovas. Pharmacol. 27: 845-853, 1996.
10. Li Z, Liu S, Qian G, Li P. Determination of the content of icariine in four Chinese patent medicines containing *Epimedium brevicornum* Maxim (Chinese). Hua-Hsi i Ko Ta Hsueh Hsueh Pao (J. West China University Med. Sci.). 26: 66-69, 1995.

11. Marin J, Rodriguez-Martinez MA. Role of vascular nitric oxide in physiological and pathological conditions. Pharmacol. Therap. 75: 111-134, 1997.
12. Fujita S, Roerig DL, Bosnjak ZJ, Stowe DF. Effects of vasodilators and perfusion pressure on coronary flow and simultaneous release of nitric oxide from guinea pig isolated hearts. Cardiovas. Res. 38: 655-667, 1998.
13. Peng W, Hoidal JR, Farrukh IS. Role of a novel K_{Ca} opener in regulating K^+ channels of hypoxic human pulmonary vascular cells. Am. J. Respir. Cell Mol. Biol. 20: 737-745, 1999.
14. Jackson WF. Ion channels and vascular tone. Hypertension 35: 173-178, 2000.
15. Sobey CG. Potassium channel function in vascular disease. *Arterioscl. Throm. Vas. Biol.* 21: 28-38, 2001.
16. Adeagbo A, Triggle C. Varying extracellular [K^+]: a functional approach to separating EDHF-and EDNO-related mechanisms in perfused rat mesenteric arterial bed. J. Cardiovas. Pharmacol. 21: 423-429, 1993.
17. Dhein S, Pejman P, Krusemann K. Effects of the $I_{(K.ATP)}$ blockers glibenclamide and HMR1883 on cardiac electrophysiology during ischemia and reperfusion. Eur. J. Pharmacol. 398, 273-284, 2000.
18. Wang MQ, Bi ZM, Li P, Ji H, Cheng FL. Determination of epimedin C and icariin in Herba Epimedii by HPLC. Zhongguo Zhong Yao Za Zhi 28: 1025-1027, 2003.

J Chin Med 17(1-2): 75-84, 2006

淫羊藿水抽提物舒張離體大白兔胸主動脈之活性研究

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(95 年 6 月 13 日受理，95 年 7 月 25 日接受刊載)

本研究探討淫羊藿水抽提物（water extract of *Epimedium brevicornum* Maxim; EB-W）舒張離體大白兔胸主動脈之機轉。結果顯示 EB-W 在 0.001-1 mg/mL 的濃度範圍內呈現劑量相關性地拮抗腎上腺素（phenylephrine）引起的血管收縮反應。此血管舒張反應可以明顯地，但不完全地，因爲去除血管內膜、預處理 N^G-nitro-L-arginine methyl ester）一氧化氮合成酶抑制劑）及 1-*H*-[1,2,4] oxadiazolo[4, 3-α]quinoxalin-1-one（guanylate cyclase 抑制劑）而受到抑制，顯示 EB-W 透過部份內膜依賴性的機轉產生舒張血管活性。而 EB-W 在去除內膜血管的殘餘舒張反應可因爲曝露於高鉀(60 mM KCl)溶液中及預處理 ATP-敏感性的鉀離子通道阻斷劑(glibenclamide)而完全被抑制。綜合以上結果顯示淫羊藿水抽提物可透過刺激血管內膜釋放一氧化氮及活化血管平滑肌上的 ATP-敏感性鉀離子通道而產生舒張活性。

關鍵詞：淫羊藿，一氧化氮，鉀離子通道，胸主動脈，血管舒張。

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