대한침구학회지 제24권 제2호(2007년 4월)
The Journal of Korean Acupuncture & Moxibustion Society

원 저

Anti-platelet Effect of Carvacrol Extracted from *Thuja Orientalis L.* : A Possible Mechanism Through Arachidonic Acid Pathway

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국문초록

백자인에서 추출된 Carvacrol의 항혈소판 효과

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목적 : 백자인에서 추출된 Carvacrol이 혈소판 활성화와 혈액 응고에 미치는 영향에 대해 알아보고자 하였다.

방법 : Carvacrol의 항혈소판 효과의 기제를 밝히기 위해 토끼 혈소판으로 Arachidonic Acid 유리, TXB2, PGD2, 12-HETE의 생성을 방사선 크로마토그래피 분석을 사용하여 측정하였다.

결과 : 1. U46619를 제외하고 Collagen과 AA에 의해 유발된 응고는 Carvacrol 농도에 따라 억제되었다.
2. Collagen으로 인하여 자극된 AA 유리에 대한 Carvacrol의 유의한 억제 효과는 나타나지 않았다.
3. AA로 유발된 TXB2, PGD2와 12-HETE의 생성 억제에 대한 실험에서 Carvacrol은 유의한 억제가 있는 것으로 나타났으며, 농도의존적으로 억제되었다.

결론 : Carvacrol은 항혈소판 작용이 있는 것으로 볼 수 있다. 이는 한의학에서 활혈거어 작용으로 해석될수 있으며, 타박상, 월경곤란증, 탈모증 등 어혈 질환의 예방및 치료와 관련된 약침 개발에 기초가 될 수 있을 것으로 사료된다.

· Acceptance : 2007년 3월 5일 · Adjustment : 2007년 3월 22일 · Adoption : 2007년 1월 12일
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핵심단어 : huja orientalis L., Carvacrol, Anti-platelet, Arachidonic acid, Thromboxane A2, prostaglandin D2, 12-hydroxyeicosatetraenoic acid

Ⅰ. Introduction

Platelet aggregation is a complex phenomenon that probably involves several intracellular biochemical pathways. When activated, platelets change shape, aggregate and release the contents of their intracellular granules[1]. The interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases[2-4]. When blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where blood flow is disturbed. These thrombi are the source of thromboembolic complications of atherosclerosis, heart attacks, stroke, and peripheral vascular disease[5]. Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis. Platelets are activated by a number of physiological agonists such as collagen, arachidonic acid(AA), thrombin or platelet activating factor(PAF), and undergo a complex cascade of events that results in shape change, secretion, formation of AA metabolites, and aggregation[6]. Carvacrol is a primary constituent of the essential oil extracted from an evergreen species, Thuja orientalis L. which grows naturally in China, Korea, Japan, and is usually used for ameliorating such symptoms induced by insufficiency of heart blood as insomnia, constipation, dizziness, palpitation and amnesia in korean traditional medicine[7,8]. It possesses antifungal activity against vaginal and oral candidiasis induced by Candaida albicans[9,10] and antibacterial activities to E. coli O157:H7 and Salmonella enterica[11]. It has also showed inhibitory effect on DNA synthesis in mouse myoblast cells bearing a human N-RAS oncogene[12] and significantly decreased the serum cholesterol levels by induction of geranyl pyrophosphate pyrophosphatase activity[13].

Recently, a resemble monoterpenoid of carvacrol, eugenol, and its homologues have been known as potent antiplatelet agents inhibiting increase of intracellular Ca2+ caused by collagen, epinephrine, ADP and AA[14]. They also prevent PAF and ethanol-induced gastric mucosal damage[15]. Thus, we conducted a preliminary screening for determination of anti-platelet activities of monoterpenoids. Carvacrol from Thuja orientalis L. was the most potent anti-platelet compound among the tested compounds and its inhibitory effect on platelet aggregation has been investigated. Therefore, we investigated whether and how carvacrol from Thuja orientalis L. had influence upon platelet aggregation and blood coagulation, while we expected carvacrol from Thuja orientalis L. to be helpful and available for prevention or treatment of static blood-related diseases via activating blood and resolving stasis.

Ⅱ. Materials and methods

1. Materials

Carvacrol, 5-isopropyl-2-methylphenol, was obtained from Aldrich Co. INC. (Milwaukee, WI, U.S.A). Collagen and arachionic acid were obtained from Chrono-Log Co. (Havertown, PA, U.S.A.). U46619 (9,11-dideoxy-9a,11 a-methanoepoxy-prostaglandin F2a), TXB2, PGD2 and 12-HETE were from Cayman Chemical Co. (Ann Arbor, MI, USA). Indomethacin and imidazole were from Sigma

Chemical Co. (St Louis, MO, USA). TXB2 enzyme immunoassay kit was purchased from Amersham Pharmacia Biotech(Little Chalfont, Buckinghamshire, UK). [3H]AA(100μCi/mmol) was from Perkin-Elmer Life Sciences Inc. (Boston, MA, USA). Other chemicals were of analytical grade.

2. Animals

Male white rabbits were purchased from Samtako Bio Korea Inc. (Osan, Gyunggi, Korea) and acclimated for at least 1week at a temperature of 24 ± 1°C and a humidity of 55 ± 5%. The animals had free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Kangwon, Korea) and drinking water before experiments. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Chungbuk National University, Korea.

3. In vitro platelet aggregation measurement

The platelet aggregation in vitro was performed as previously described[16]. In brief, rabbit blood was collected from the ear aorta with a one-tenth volume of 1% EDTA. Platelet-rich plasma (PRP) was obtained by centrifugation at 230 × g for 10 min. Platelets were sedimented by centrifugation of the PRP at 800 × g for 15min, and then washed twice with HEPES buffer(137mM NaCl, 2.7mM KCl, 1mM MgCl2, 5.6mM glucose and 3.8 mM HEPES, pH 6.5) containing 0.35% bovine serum albumin and 0.4 mM EGTA. The washed platelets were resuspended in HEPES buffer (pH 7.4). The platelet number was counted by Coulter Counter(Coulter Electronics, Hialeah, FL, USA) and adjusted to a concentration of 3 × 108 platelets/ml. Washed rabbit platelets were incubated at 37°C for 3min with various concentrations of carvacrol in the presence of 1 mM CaCl2 in an aggregometer(470-vs, Chrono-log Co., PA, USA), and then platelet aggregation was induced by addition of collagen (1μg/ml), AA (100 μM) or U46619(1μM). The resulting aggregation, measured as the change of light transmission, was recorded for 10min. Each inhibition rate was obtained from the maximal aggregation induced by respective agonist at the concentration using the equation: inhibition RATE=(maximal aggregation rate(MAR) of vehicle- treated PRP-MAR of sample-treated PRP/MAR of vehicle-treated PRP) × 100. The values of IC50(50% inhibition concentration) were calculated from the data using a probit method.

4. AA liberation determination

The effect of carvacrol on AA liberation stimulated by collagen in [3H]AA-labeled rabbit platelets was assayed as done previously[16]. In brief, washed rabbit platelets(3×108 platelets/ml) were preincubated with [3H]AA (1μCi/ml) at 37°C for 1.5h, and then washed as described above. The [3H]AA-labeled platelets were pretreated with 100 MBW755C, a COX and LOX inhibitor, and various concentrations of carvacrol at 37°C for 3 min in the presence of 1mM CaCl2, and then stimulated with collagen (50μg/ml). The reaction was terminated by addition of chloroform/methanol/HCl (200 : 200 : 1, v/v/v). Lipids were extracted and separated by TLC on silica gel G plates with the following development system: petroleum ether/diethyl ether/acetic acid (40 : 40 : 1, v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

5. TXB_2, PGD_2 and 12-HETE generation determination

The effects of carvacrol on TXB_2, PGD_2 and 12-HETE generations were assayed as done previously[16]. Washed rabbit platelets(3×108 platelets/ml) were preincubated with various concentrations of carvacrol at 37°C for 3min, and then further incubated with a mixture of [3H]AA

and unlabeled AA(1μCi/ml, 2μM) for 5min. The reaction was terminated by addition of stop solution(2.6mM EGTA, 130 MBW755C). Lipids were extracted and separated by TLC on silica gel G plates with the following development system: ethyl acetate/isooctane/acetic acid/H2O(9 : 5 : 2 : 10, v/v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

6. Statistical analysis

The experimental results were expressed as the means ± SEM. Data were analysed using one-way ANOVA followed by Dunnett's test, and a probability value less than 0.05 was considered as statistically significant.

Ⅲ. Results

1. Effect of carvacrol on platelet aggregation in vitro

Washed rabbit platelets were preincubated with various concentrations of carvacrol(3, 5, 10, 30, and 60μM), and then were exposed to collagen (1μg/ml), AA (100μM) or U46619 (1μM), to examine the inhibitory effect of carvacrol on rabbit platelet aggregation. As shown in Fig. 1, carvacrol concentration-dependently inhibited the aggregations induced by collagen and AA, except for U46619. The IC50 values of carvacrol on collagen-, AA-induced rabbit platelet aggregation were 12.6 ± 1.1 and 2.5 ± 0.5μM, respectively.

2. Effect of carvacrol on AA liberation

The effect of carvacrol on AA liberation stimulated by collagen in platelets was assayed by using [3H]AA-labeled rabbit platelets. When [3H]AA-labeled platelets were incubated with carvacrol (5, 10, 30 and 60μM) at 37°C for 3min and then exposed to 50μg/ml collagen, carvacrol did not suppress collagen-induced AA liberation from [3H]AA-labeled platelets.(Fig. 2). AA liberation of carvacrol 5, 10, 30 and 60 were 7490 ± 542, 7570 ± 509, 7090 ± 386 and 7010 ± 267μM, respectively. The result indicated that it has no effect on PLA2 activation in response to collagen compared with control.

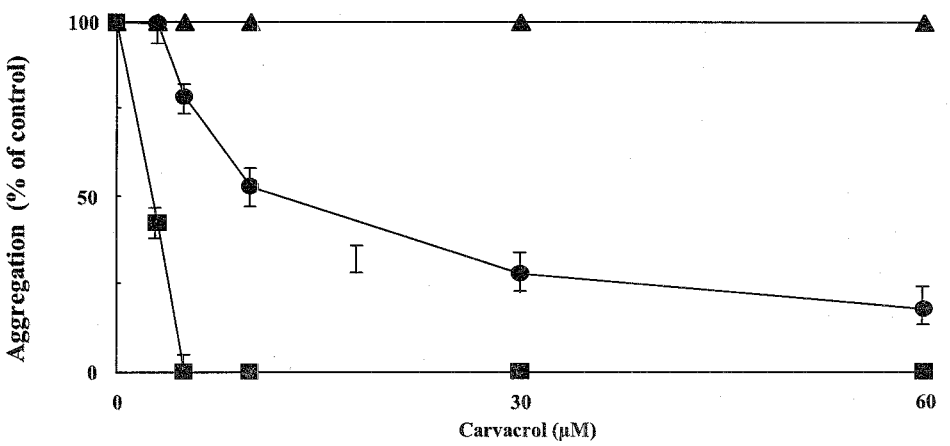

Fig. 1. Effect of Carvacrol on Rabbit Platelet Activation Induced by Collagen, Arachidonic Acid, or U46619. Washed platelets were preincubated with various concentrations of carvacrol for 3 min in the presence of 1 mM $CaCl_2$, and stimulated with 1 μg/mL collagen(●), 100 μM arachidonic acid(■), and 1 μM U46619(▲) in an aggregometer. The change in light transmission of the platelet suspension after 10 min stimulation with each agonist alone was taken as 100%.

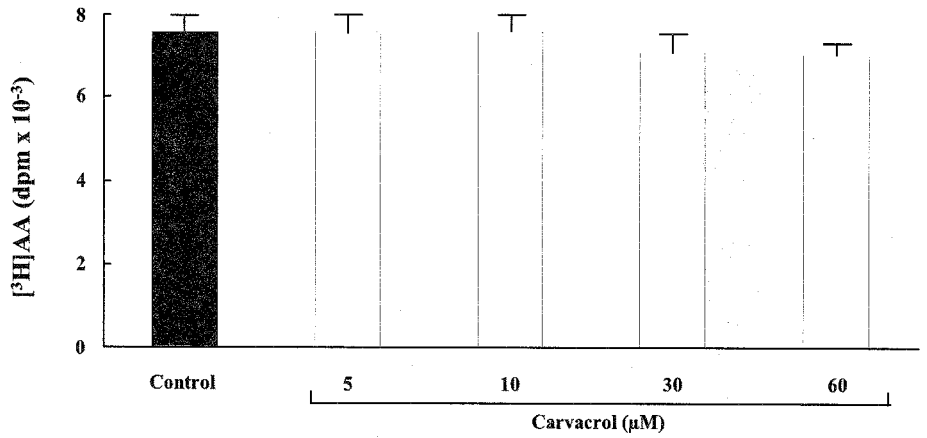

Fig. 2. Inhibition by Carvacrol of Arachidonic Acid Liberation Induced by collagen. **[^{3}H]AA-labeled platelets were incubated with various concentrations of carvacrol at 37°C for 3 min in the presence of 50 μM BW755C and 1 mM $CaCl_2$, and then stimulated with 50 μg/mL collagen for 2 min. [^{3}H]AA liberated was determined as described in Materials and Methods.**

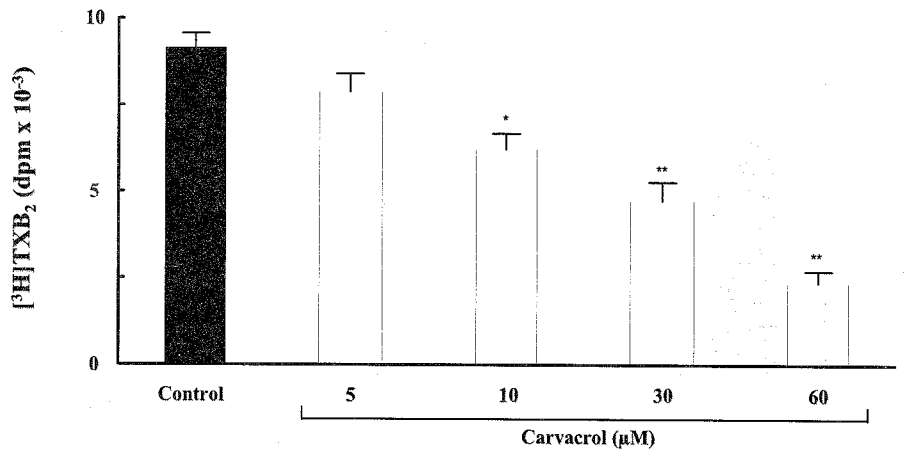

Fig. 3. Effects of Carvacrol on Conversion of Arachidonic Acid to TXB_2 in Intact Platelets. **Washed platelets were preincubated with various concentrations of carvacrol for 3 min without $CaCl_2$, and further incubated with a mixture of [^{3}H]AA and the unlabeled compound (2 μM) for 5 min. The [^{3}H]TXB_2 generated was measured as described under Materials and Methods. * represents significantly different from control, P<0.05 and **represents significantly different from control, P<0.01.**

3. Effects of carvacrol on TXB_2, PGD_2, and 12-HETE generations

In order to determine the effects of carvacrol on the conversion of exogenously added AA to metabolites of COX pathway(including TXB2 and PGD_2) and 12-LOX pathway(12-HETE), washed rabbit platelets were pre-incubated with various concentrations of carvacrol(5, 10, 30 and 60μM), and then further incubated with the mixture of [3H]AA and unlabeled AA(1μCi/ml, 2M). Carvacrol significantly suppressed the TXB_2, PGD_2 and 12-HETE formation induced by addition of [3H]AA in intact washed rabbit platelets in dose-dependently manner. TXB_2 sinificantly decreased by 10, 30 and 60μM of Carvacrol was 6240 ± 541, 4730 ± 639 and 2350 ± 406dpm(Fig. 3), and PGD_2 sinificantly decreased by 30 and 60 μM of Carvacrol was 5230 ± 517 and 1450 ± 698 dpm (Fig. 4), and 12-HETE sinificantly decreased by 60μM of Carvacrol was 7620 ± 2309 dpm(Fig. 5).

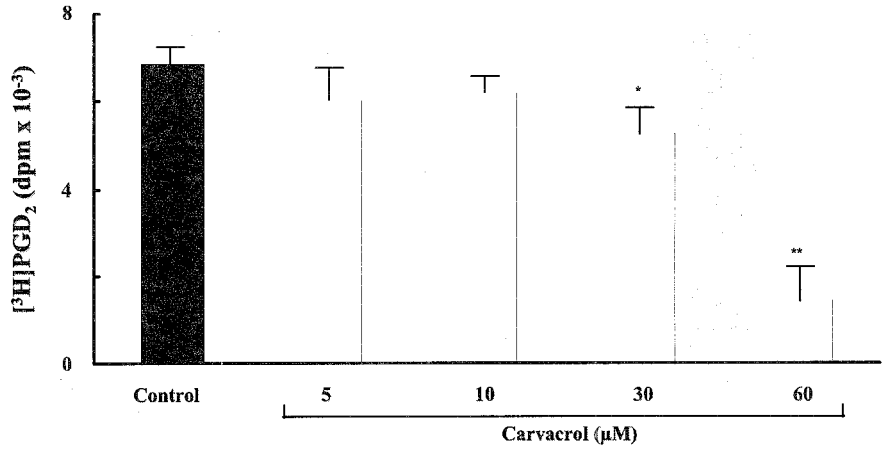

Fig. 4. Effects of Carvacrol on Conversion of Arachidonic Acid to PGD_2 in Intact Platelets. Washed platelets were preincubated with various concentrations of carvacrol for 3 min without $CaCl_2$, and further incubated with a mixture of [^{3}H]AA and the unlabeled compound (2 μM) for 5 min. The [^{3}H]PGD_2 generated was measured as described under Materials and Methods.

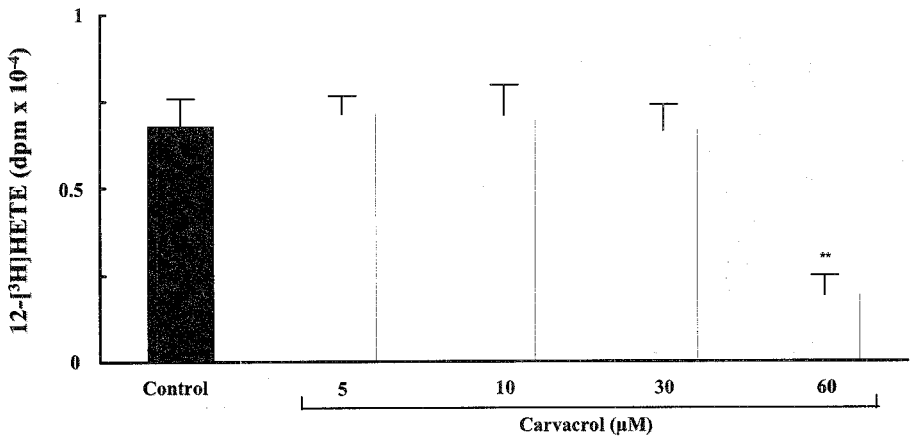

Fig. 5. Effects of Carvacrol on Conversion of Arachidonic Acid to 12-HETE in Intact Platelets. Washed platelets were preincubated with various concentrations of carvacrol for 3 min without $CaCl_2$, and further incubated with a mixture of [^{3}H]AA and the unlabeled compound (2 μM) for 5 min. The [^{3}H]12-HETE generated was measured as described under Materials and Methods.

Ⅳ. Discussion

Thuja orientalis L. has effect of nutrition and stability, is good at such symptoms induced by insufficiency of heart blood as insomnia, constipation, dizziness, palpitation.

The objective of this study was determined that the effect of carvacrol on platelet aggregation and AA metabolism. To elucidated a possible mechanism of antiplatelet effect of carvacrol, we investigated AA liberation, formation of TXB_2 and PGD_2, through COX pathway, or 12-HETE, through LOX pathway [3H]AA using radio-chromatographic analysis with washed rabbit platelets in vitro, and evaluated TXA_2 synthase activity.

As the results demonstrated, carvacrol mainly inhibited the rabbit platelet aggregation induced by AA in a dose-dependent manner, while that induced by collagen was slightly inhibited at the concentration tested. However, carvacrol had not shown any anti-platelet activity stimulated by

U46619(Fig. 1). Similarly, a methanol soluble fraction of black cumin oil prepared from seeds of Nigella sativa suppressed on AA-induced platelet aggregation and blood coagulation. Then, a new compound 2-(2-methoxypropyl)-5-methyl-1,4-benzenediol and two known compounds, thymol and carvacrol were isolated from the methanol fraction of the essential oil of cumin. These compounds possessing aromatic hydroxyl and acetoxyl group had more potent activity than aspirin, which is well known as a remedy for thrombosis[17].

Several agonists evoke the rapid liberation of AA from membrane phospholipids. Since AA is a precursor of prostaglandin endoperoxides, TXA2 and other eicosanoids, which also mediate platelet activation, AA liberation is a key step in signal transduction[18]. Since the intracellular concentration of free AA is low, the liberation of AA is thought to be the rate-limiting step in the formation of PGs and the other eicosanoids, including TXA2 in platelets[19]. There are many reports that show inhibitory effects on platelet aggregation by a variety of agents inhibiting AA liberation, such as PLA2[20]. In the present study, we examined the effect of carvacrol on AA metabolism for elucidating a possible anti-platelet mechanism of it through AA libration from membrane phospholipids in AA-stimulated platelet pre-labeled with [3H]AA and we found that carvacrol did not significantly affect AA libration, the result indicated platelet aggregation activity of carvacrol is possibly not directly due to interference with PLA2(Fig. 2).

Platelets are activated by a number of physiological agonists such as ADP, thrombin, collagen, platelet activating factor (PAF), TXA2, and undergo a complex cascade of events that results in shape change, secretion, formation of AA metabolites, and aggregation. In fact, collagen, evokes platelet shape change, the release reaction, phospholipase C(PLC) activation, PLA2 activation, and aggregation. On the other hand, several lines of evidence suggest that two metabolic pathways of AA liberated by Ca2+-dependent PLA2 exist in platelets. One is the COX pathway that forms TXA_2 and PGD_2, and the other is the LOX pathway that forms 12-HETE. The significance of the COX product TXA_2 is well-recognized, but the role of LOX products has not been clear. Different enzymatic pathway may function to mobilize AA from platelet phosphatides. The physical perturbation, such as vessel injury or decreased membrane fluidity, may influence the stimulus-induced changes in platelet lipids leading to liberation of AA. This AA is converted to TXB_2 and PGD_2, through COX pathway, or 12-HETE, through LOX pathway. Our data demonstrated that Carvacrol significantly inhibited the TXB_2, PGD_2 and 12-HETE generations induced by addition of AA in intact washed rabbit platelets(Fig. 3, 4, 5), which suggested that anti-platelet activity of carvacrol may be related to the inhibition on COX and LOX pathway in platelet aggregation via concentration-dependent suppression of AA-derived metabolites.

In conclusion, Anti-platelet activity of carvacrol in the present study could be translated into activity of activating blood and resolving stasis in the concept of traditional korean medicine and confirm a basis for the development of carvacrol from Thuja orientalis L. as a novel pharmaco-acupuncture agent for prevention and/or intervention of static blood-related diseases including contusion, dismenorrhea, alopecia, etc.

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