Inhibitory effects of Cinnamomi Cortex and cinnamaldehyde on oxygen-derived free radical-induced vasocontraction in isolated aorta of spontaneously hypertensive rats

Yuji Kasahara,[*a] Hirozo Goto,[b] Yutaka Shimada,[a] Nobuyasu Sekiya,[a]
Qiao Yang,[a] and Katsutoshi Terasawa[a]

[a]*Department of Japanese Oriental (Kampo) Medicine, Toyama Medical and Pharmaceutical University,*

[b]*Department of Kampo Diagnostics, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University,*

2630 Sugitani, Toyama 930-0194, Japan.

Abstract

We examined the inhibiting effect of Cinnamomi Cortex extract (CCE) and cinnamaldehyde (CA) against vasocontraction induced by oxygen-derived free radicals produced by the xanthine-xanthine oxidase (XOD) system in the thoracic aortic ring of the spontaneously hypertensive rat (SHR), using the organ bath method *in vitro*.

The vasocontraction induced by xanthine-XOD in the CCE (10^{-4} g/ml) and CA (10^{-4} M) treatment groups were significantly lower than that in the control group. Further, the amounts of thromboxane B_2 (TXB_2) produced in the vasocontractive response in the CCE (10^{-4} g/ml) and CA (10^{-4} M) treatment groups were significantly lower than that in the control group.

For the purpose of examining the mechanism of the inhibiting effect of CA against thromboxane production, the inhibiting effect of CA against the vasocontraction induced by phospholipase A_2 (PLA_2) was examined. The vasocontraction induced by PLA_2 in the CA (10^{-4} M) treatment group was significantly lower than that in the control group. Moreover, the amount of TXB_2 produced by the vasocontractive response in the CA (10^{-4} M) treatment group was significantly lower than that in the control group.

From the above findings, it is suggested that Cinnamomi Cortex is an agent which exerts an inhibitory effect on the vasocontractive factor (TXA_2) *in vitro*.

Key words Cinnamomi Cortex, cinnamaldehyde, vasocontraction, spontaneously hypertensive rats, phospholipase A_2, thromboxane A_2, endothelial dysfunction.

Abbreviations CA, cinnamaldehyde; CCE, Cinnamomi Cortex extract; KB, Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan), 桂枝茯苓丸; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; PLA_2, phospholipase A_2; SHR, spontaneously hypertensive rat; SOD superoxide dismutase; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; WKY, Wistar-Kyoto rat; XOD, xanthine oxidase.

Introduction

Vascular endothelial cells produce and secrete various vascular relaxing factors such as nitric oxide (NO),[1] prostaglandin I_2,[2] and endothelium-derived hyperpolarizing factor as well as contracting factors such as endothelin and thromboxaneA_2 (TXA_2).[3] These factors influence each other to create a balance, they maintain a vascular tonus, and they are involved in blood pressure regulation.[1] There have been many reports concerning the fact that hypertension and vascular endothelial dysfunction are closely related.[4,5] Vascular endothelial dysfunction stimulates remodelling of the vascular structure from both a decrease of relaxing factors[6] and an increase of contracting factors.[7] Arteriosclerosis progresses, and finally internal organ disorder results.

In our previous study, we have reported on the pro-

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*To whom correspondence should be addressed.

tective effect of Keishi-bukuryo-gan (KB; Gui-Zhi-Fu-Ling-Wan) against vascular endothelial disorder in spontaneously hypertensive rats (SHR).[8] We have found that the contraction induced by xanthine-xanthine oxidase (XOD) is significantly lower in the KB group than in the control group.

The contractive response induced by xanthine-XOD is considered to represent a disordered vascular model caused by arteriosclerosis. The participation against this response of an increase in the production of free radicals in the vascular endothelium and TXA_2, a kind of contracting factor, is reported.[9]

It is reported that Cinnamomi Cortex, the main component of KB, induces endothelial-dependent vascular relaxation[10] and has an improving effect on blood circulation.[11] However, the effect of Cinnamomi Cortex on the vasocontractive response has not been confirmed.

In this study, we examine the effect of Cinnamomi Cortex extract (CCE) on the contraction induced by oxygen-derived free radicals produced by the xanthine-XOD system and on TXA_2 production *in vitro*.

Materials and Methods

Preparation of extracts and chemicals: Keihi (桂皮), Cinnamomi Cortex, *Cinnamomum cassia* BLUME, (China), were purchased from Tochimoto Tenkaido (Osaka, Japan). The extract was obtained by boiling these materials in water for 50 minutes and then freeze-drying into a resultant powder. We obtained 3.0g of CCE from 100 g of the raw materials. The CCE powder was then dissolved in distilled water for the experiments. KCl, indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), superoxide dismutase (SOD) from bovine erythrocytes, dimethyl sulfoxide, xanthine and XOD in suspension were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phospholipase A_2 (PLA_2) from bee venom (Apis mellifera) and catalase from bovine liver were purchased from SIGMA-ALDRICH JAPAN K.K. (Tokyo Japan). Trans-cinnamaldehyde was purchased from TOKYO KASEI KOGYO CO., LTD. (Tokyo Japan). Indomethacin and CA were dissolved in dimethyl sulfoxide, and other reagents were dissolved in distilled water and used.

Test animals: SHR and Wistar-Kyoto rats (WKY; 15-20 weeks old, male) were purchased from Sankyo

Labo Service (Tokyo, Japan). Experimental protocols met the "Guidelines for Animal Experimentation" approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

Contraction experiments: The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and killed by drawing blood from the heart. A section of the thoracic aorta was carefully cleaned by removing fat and connective tissues, and ring preparations (3 mm wide) were prepared. The rings were mounted on steel hooks in a Magnus chamber (Kishimoto UC-5TD, Kyoto, Japan). One end of the aorta was attached to a force-displacement transducer (Kishimoto UM-203), and its isometric contraction was recorded (Niko Bioscience T-634, Tokyo, Japan). Baths were filled with 7 ml of Krebs solution with the following composition (mM): NaCl 120, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, and glucose 10.0. The solution was maintained at 37°C and bubbled continuously with 5% CO_2 - 95% O_2 at pH 7.4. The rings were equilibrated for 40 min at an initial resting tension of 1 g, and then contracted with 60 mM KCl. When contraction reached a steady maximal response, 10^{-6} M acetylcholine was added. Then the Krebs solution was replaced every 15 minutes for 60 minutes. Finally, the preparations with the endothelium intact were exposed to L-NAME (10^{-4} M) for 60 minutes before contraction.

a) Contraction induced by xanthine-XOD: After it was confirmed that contraction returned to the baseline, each aortic strip was pretreated with CCE (10^{-4}-10^{-6} g/ml); the main component of Cinnamomi Cortex, CA (10^{-4} M-10^{-6} M); cyclooxygenase inhibiter, indomethacin (10^{-5} M); superoxide anion scavenger, SOD (150 U/ml); and hydrogen peroxide catabolic enzyme, catalase (1000 U/ml). At 10 minutes after the addition of the pretreatment drugs, pretreatment with xanthine (10^{-4} M) was performed, and 10 minutes after that, XOD (10 mU/ml) was added. Transient contraction was thereby induced. The contraction was expressed as a percentage of 60 mM KCl maximum contraction.

b) Contraction induced by xanthine-XOD with indomethacin: After it was confirmed that contraction returned to the baseline, each aortic strip was pretreated with indomethacin (10^{-5} M). At 10 minutes after its addition, each aortic strip was pretreated with CCE (10^{-4}

g/ml), CA (10^{-4} M) or SOD (150 U/ml). At 10 minutes after the addition of pretreatment drugs, xanthine (10^{-4} M) was added, and XOD (10 mU/ml) was added 10 minutes later. This procedure induced transient contraction. The contraction was expressed as a percentage of the maximum contraction at 60 mM KCl.

c) Contraction induced by PLA2: To examine the effect of CA against TXA_2-induced contraction, PLA_2 (1 U/ml) was administered at 10 minutes after the addition of pretreatment drugs, and transient contraction was induced. The contraction was expressed as a percentage of 60 mM KCl maximum contraction.

Thromboxane B_2 (TXB_2) measurement: In the a) and c) contraction experiments, organ-chamber solutions (2 ml) were collected, respectively, before xanthine pretreatment, 30 minutes after XOD treatment, just before PLA_2 administration, and 30 minutes after PLA_2 administration, and they were used for TXB_2 measurement. TXA_2 is an extremely unstable molecule and rapidly undergoes hydrolytic degradation to stable TXB_2,[12] which was used as an index of *in vivo* TXA_2 generation. TXB_2 concentration in bath solution was measured by RIA PEG method.[13]

Statistical analysis: Data were presented as mean $\pm$ standard error. Statistical comparisons were done by Mann-Whitney test and repeated measures ANOVA, followed by Fisher's PLSD for multicomparisons, and all values were compared with SHR control values. The level of statistical significance was defined as $p < 0.05$.

Results

Contraction induced by oxygen-derived free radicals produced by the xanthine-XOD system in SHR and WKY

Xanthine (10^{-4} M) plus XOD (10 mU/ml) induced a temporary contraction of the aortic strips with L-NAME. The typical contraction responses are shown in Fig.1A. Contraction of SHR group induced by xanthine-XOD was 73.7 $\pm$ 3.19% (n = 10), and that of the WKY group was 11.4 $\pm$ 3.58% (n = 4); that of the indomethacin-treated (10^{-5} M) SHR group was 9.32 $\pm$ 3.30% (n = 10), that of the SOD-treated (150 U/ml) SHR group was 40.2 $\pm$ 11.01% (n = 10), and that of the catalase-treated (1000 U/ml) SHR group was 27.0 $\pm$ 6.25% (n = 4; Fig.2). The contractions were significantly greater in the SHR control group than in the WKY group. Both free
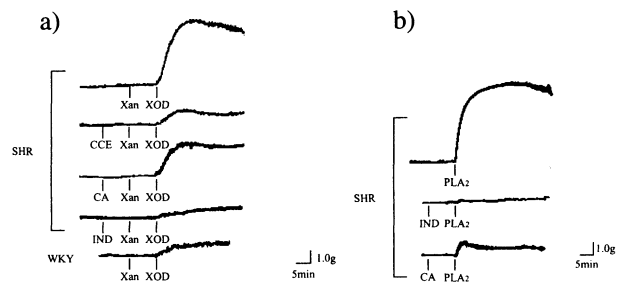

Figure 1. Typical tracings of vasocontraction of isolated SHR and WKY aortas after reagent (10^{-5} M indomethacin (IND), 10^{-4} g/ml Cinnamomi Cortex extract (CCE) or 10^{-4} M cinnamaldehyde (CA)) treatment.
a) Ring contracted with xanthine oxidase (XOD; 10 mU/ml) in the presence of xanthine (Xan; 10^{-4} M).
b) Ring contracted with phospholipase A_2 (PLA_2;1 U/ml).
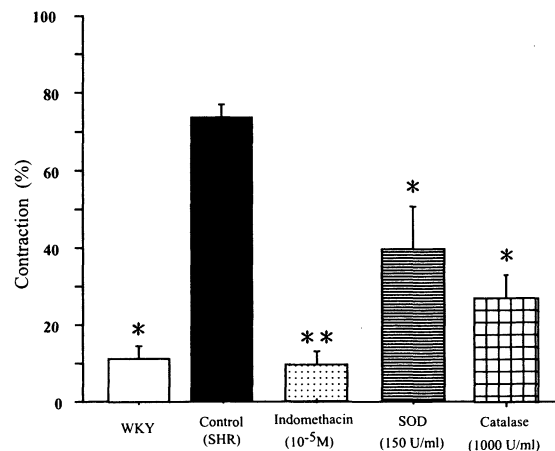All aortas had intact endothelium, and had been treated with L-NAME.

Figure 2. Inhibitory effects of reagent (10^{-5} M indomethacin, 150 U/ml SOD or 1000 U/ml catalase) treatment on XOD (10 mU/ml) in the presence of xanthine (10^{-4} M) - induced vasocontraction in isolated SHR and WKY aortas. All aortas had intact endothelium, and had been treated with L-NAME. Contraction was expressed a percentage of 60 mM KCl maximum contraction. Asterisks indicate significant differences from SHR control group. (*$p < 0.05$; **$p < 0.01$, mean $\pm$ S.E., n = 4~10)

radical scavengers (SOD and catalase) and cyclooxygenase inhibitor (indomethacin) inhibited the contraction significantly.

Effects of CCE and CA against contraction induced by oxygen-derived free radicals produced by the xanthine - XOD system in SHR

The contraction of the SHR group induced by xanthine-XOD was 73.7 $\pm$ 3.19%, that of the CCE-treated (10^{-4} g/ml) SHR group was 25.8 $\pm$ 5. 19%, and that of the CA-treated (10^{-4} M) SHR group was 31.3 $\pm$ 7.63%. Both CCE (10^{-4} g/ml) and CA (10^{-4} M) inhibited the contractions significantly, and both inhibited the con-
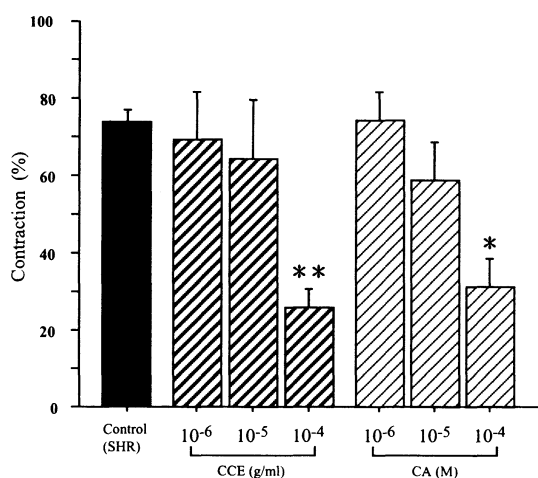
Figure 3. Inhibitory effects of reagent (10^{-6} -10^{-4} g/ml CCE or 10^{-6} - 10^{-4} M CA) treatment on XOD (10 mU/ml) in the presence of xanthine (10^{-4} M) induced vasocontraction in isolated SHR aortas. All aortas had intact endothelium, and had been treated with L-NAME. Contraction was expressed a percentage of 60 mM KCl maximum contraction. Asterisks indicate significant differences from SHR control group. ($*p < 0.05$; $**p < 0.01$, mean $\pm$ S.E., n = 6~10)
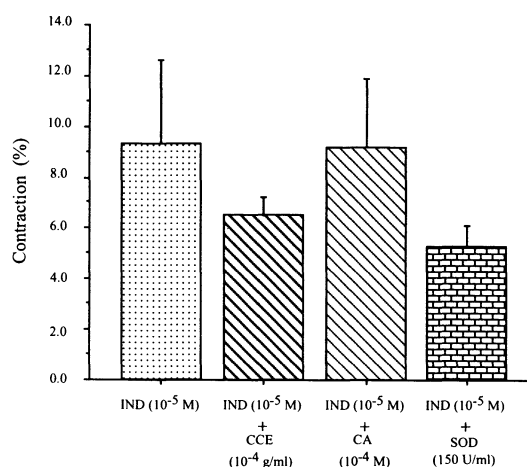

Figure 4. Inhibitory effects of the reagent (10^{-4} g/ml CCE, 10^{-4} M CA or 150 U/ml SOD) treatment on XOD (10 mU/ml) in the presence of xanthine (10^{-4} M) - induced vasocontraction in isolated SHR aortas. All aortas had intact endothelium, and had been treated with L-NAME and 10^{-5} M indomethacin. Contraction was expressed a percentage of 60 mM KCl maximum contraction. (mean $\pm$ S.E., n = 4~10)

Table I TXB_2 concentration in organ-chamber solution (pg/ml)

Vasocontraction inducer	xanthine (10^{-4} M)-XOD (10 mU/ml)		PLA₂ (1U/ml)	
	before	after	before	after
Control(SHR)	9.84 ± 1.63	$91.1 \pm 11.9*$	17.6 ± 7.61	$77.6 \pm 14.9*$
CCE (10^{-4} g/ml)	12.5 ± 1.19	$34.1 \pm 3.73*^#$	$(-)$	$(-)$
Cinnamaldehyde (10^{-4} M)	9.76 ± 2.45	$47.2 \pm 6.98*^#$	7.80 ± 1.96	$29.0 \pm 3.94*^#$

Changes in the concentration of TXB_2 in organ-chamber solution in SHR aortic rings in the vasocontraction inducer after reagent (CCE or CA) treatment. All aortas had intact endothelium, and had been treated with L-NAME. $*p<0.05$, vs. before vasocontraction. $#p<0.05$, vs. SHR control group (mean$\pm$S.E., n=4~7).

tractions in a concentration-dependent manner (each group: n = 6-10; Fig.3).

Effects of CCE and CA against TXB_2 production in the xanthine-XOD-induced contraction

The TXB_2 concentration of the bath solution at 30 minutes after vasocontraction by xanthine-XOD of SHR in the CCE-treated (10^{-4} g/ml) group was significantly lower than that in the control group. The CA (10^{-4} M) group showed a similar result (n = 6-7; Table I).

Contraction induced by oxygen-derived free radicals produced by the xanthine-XOD system in SHR with indomethacin

Contraction of the SHR group with L-NAME and indomethacin induced by xanthine-XOD was 9.4 $\pm$ 3.30% (n = 10) that of the CCE-treated (10^{-4} g/ml) SHR group was 6.5 $\pm$ 0.71% (n = 10), that of the CA-treated (10^{-4} M) SHR group was 9.2 $\pm$ 2.74% (n = 6), and that

of the SOD-treated (150 U/ml) SHR group was 5.3 $\pm$ 0.84% (n = 4; Fig.4). In both the indomethacin + CCE and indomethacin + SOD groups, compared with the indomethacin only group, slight inhibition (not significant) of the contraction was seen. But in the indomethacin + CA group, inhibition of the contraction was not observed.

Effect of CA against contraction induced by PLA₂

PLA₂ (1 U/ml) induced a temporary contraction of the aortic strips. The typical contraction responses are shown in Fig.1B. The contraction induced by PLA₂ of the SHR control group was 75.1 $\pm$ 5.03%, that of the CA-treated (10^{-4} M) SHR group was 23.2 $\pm$ 8.61%, and that of the indomethacin-treated (10^{-5} M) SHR group was 18.0 $\pm$ 6.84%. Indomethacin and CA (10^{-4} M) inhibited the vasocontractive response significantly. CA inhibited the vasocontractive response in a concentration-dependent
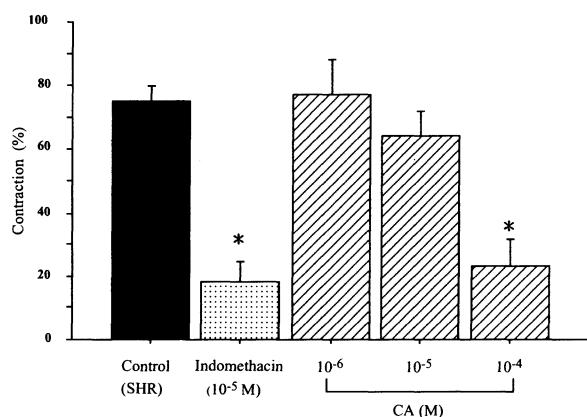
Figure 5. Inhibitory effects of the reagent (10^{-5} M indomethacin or 10^{-6}-10^{-4} M CA) treatment on PLA_2 (1 U/ml) - induced vasocontraction in isolated SHR aortas. All aortas had intact endothelium, and had been treated with L-NAME. Contraction was expressed a percentage of 60 mM KCl maximum contraction. Asterisks indicate significant differences from SHR control group. (*$p < 0.05$, mean $\pm$ S.E., n = 6~9)

manner (each group: n = 6-9; Fig.5)

Effect of CA against TXB$_2$ production in PLA$_2$-induced contraction

The TXB$_2$ concentration of the bath solution at 30 minutes after vasocontraction by PLA$_2$ of SHR in the CA-treated (10^{-4} M) group was significantly lower than that in the control group (n = 4-5; Table I).

Discussion

Cinnamomi Cortex is a clinically important crude drug that is used to improve blood circulation.[14] However, in many respects the mechanisms involved in this improvement are still unclear. One of these mechanisms was reported to be the endothelial-dependent relaxing effect of tannin present in Cinnamomi Cortex.[10] In this study, as the influence of NO was excluded by pretreatment with L-NAME, other mechanisms were studied.

It is known that vessel injury by atherosclerosis produces free radicals. Such free radicals have already been produced by combining xanthine and XOD *in vitro*.[9] When one molecule of uric acid is formed into one molecule of xanthine by XOD, one molecule of superoxide radical is also formed. It is reported that, when this superoxide radical acts on blood vessels, it causes an increase in TXA$_2$ in the blood vessels and, in turn, their contraction.[9] These phenomena were also observed in the present study (Fig.1 and 2, Table I). The contraction

induced by xanthine and XOD was significantly inhibited by pretreatment with radical scavengers (SOD and catalase; Fig.2). From these findings, it is considered that this contraction response was induced by free radicals.

The xanthine-XOD-induced contraction was inhibited by pretreatment with CCE. The increase of TXB$_2$ in the organ bath was suppressed by CCE compared to the control group (Fig.3, Table I).

TXA$_2$ is produced not only in platelets[12] but also in the endothelium and smooth muscle of vessels.[15] In this study, TXB$_2$ was considered to originate from blood vessels[16] and, as a result, TXA$_2$ production was thought to be inhibited.

Further, cinnamaldehyde, a main component of Cinnamomi Cortex, was also studied. The contraction induced by xanthine and XOD was inhibited by pretreatment with cinnamaldehyde in the same way as with CCE (Fig.3). This suggested that cinnamaldehyde was one of the main components in the inhibiting effect for xanthine and XOD contraction. Moreover, the mechanism was considered to be related to the production of TXA$_2$.

The contraction induced by xanthine-XOD was inhibited by pretreatment with indomethacin (Fig.2, Table I). It is thought that superoxide radicals stimulate the arachidonate cascade, and thus TXA$_2$ is produced. This agrees with the past report that free radical-induced contraction was inhibited by pretreatment with an inhibitor of PLA$_2$, the first enzyme of the arachidonate cascade.[17] We considered that these inhibitory effects of CCE and CA on the xanthine-XOD-induced contraction are the free radical scavenging effect, or the inhibitory effect, of the enzyme activity of a part of the arachidonate cascade. So we examined the free radical scavenging effect of CCE and CA.

The xanthine-XOD-induced contraction with indomethacin was smaller than the contraction without indomethacin (Fig.2), and was even more inhibited by pretreatment with CCE and SOD. It is suggested that this additional effect is the radical scavenging effect of SOD. Also, it is reported that the tannin present in Cinnamomi Cortex possesses a radical scavenging effect *in vitro*.[10] As for the additional effect by CCE, it is also thought to be a radical scavenging effect. On the other hand, the contraction was not inhibited by pretreatment with CA (Fig.4), and it was considered that CA does not exert a radical scavenging effect.

To determine the effect of CA on the system of TXA_2 production, we studied its effect on PLA_2-induced contraction. We found that pretreatment with CA inhibited PLA_2-induced contraction in a gradual, dose-dependent manner (Fig.5). It has been reported that CA has an inhibitory effect on thromboxane production that results from the inhibitory effect of the arachidonic acid release by the suppressive effect of PLA_2 activity on platelet aggregation in vitro.[18] Thus, it is possible that CA inhibits the PLA_2 activity on vasocontraction in vitro. It is suggested that this contraction inhibitory effect of CA is a result of its inhibitory effect on PLA_2 activity.

In earlier studies, it has been reported that drugs exerting a suppressive effect on platelet aggregation were paeonol from Moutan Radix Cortex[19] and trichosanic acid from Trichosanthis Semen.[20] But there has been no report on the effect on TXA_2 production in injured vessels.

TXA_2 has strong activity to contract vessels, so it is closely related to hypertension. KB has been reported to decrease blood pressure,[8] and this is thought to be affected by the suppression of TXA_2 production. Thus, from the present results it is suggested that CCE works to decrease blood pressure and improve related symptoms.

Further, the effects of Toki-sigyakuka-goshuyu-shokyo-to on peripheral circulation[21] and of Sai-rei-to on TXA_2 metabolism in nephrotic rat have been reported.[22] It was suggested that these effects were concerned with the suppressive effect of TXA_2 production by CA.

It has also been reported that TXA_2 has proliferating and hypertrophic effects on smooth muscle,[7] and the accumulation of the expression of mRNA of the thromboxane receptor has been recognized in the arteriosclerotic lesions of human, even in the absence of coronary disease.[23] In addition, it was reported that the serum TXA_2 concentration of a youth with genetically inherited coronary disease diathesis was elevated.[24] The accumulated evidence strongly suggests that TXA_2 is involved in arteriosclerotic development. Taken together then, the possibility that Cinnamomi Cortex has an anti-atherosclerotic effect from its suppressive effect on TXA_2 production cannot be denied.

In our past study, we have reported that tannin in Cinnamomi Cortex induced endothelial-dependent vascular relaxation in vitro.[10] When that finding and our current results are examined together, we are convincingly led to the assumption that Cinnamomi Cortex is an agent with the versatility of possessing a potent effect that manifests itself both as a vascular relaxing factor and as suppressive activity against the vasoconstrictive factor (TXA_2) in vitro.

Acknowledgments

和 文 抄 録

自然発症高血圧ラット（SHR）摘出胸部大動脈におけるキサンチン-キサンチンオキシダーゼ（XOD）誘発血管収縮反応に対する桂皮エキスおよびケイヒアルデヒド（CA）の収縮抑制作用についてオルガンバス法を用いて検討した。

キサンチン-XOD 誘発血管収縮反応は，SHR 対照群と比較して，桂皮エキス（10^{-4} g/ml）前処置群，CA（10^{-4} M）前処置群で，有意に抑制されていた。キサンチン-XOD 収縮反応時のトロンボキサン B_2（TXB_2）産生量は，SHR 対照群と比較して，桂皮エキス（10^{-4} g/ml）群，CA（10^{-4} M）群で，有意に抑制されていた。

CA の TX 産生抑制の機序を検討するため，フォスフォリパーゼ A_2（PLA_2）誘発血管収縮反応に対する収縮抑制作用について検討したところ，PLA2誘発血管収縮反応は SHR 対照群と比較して，CA（10^{-4} M）群で，有意に抑制されていた。PLA_2 収縮反応時の TXB_2 産生量は，SHR 対照群と比較して CA（10^{-4} M）群で，有意に抑制されていた。

以上のことから，桂皮は，血管収縮因子である TXA_2 抑制作用を持つ生薬である可能性が示唆された。

*〒930-0194　富山市杉谷 2630
富山医科薬科大学医学部和漢診療学講座　笠原裕司

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