Antihypertensive and Renoprotective Effects of Dietary Flaxseed and its Mechanism of Action in Deoxycorticosterone Acetate-Salt Hypertensive Rats

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Keywords

Flaxseed · Alpha-linolenic acid · Hypertension · Sympathetic nervous system · Brain inflammation

Abstract

Background/Aims: Flaxseed contains alpha-linolenic acid (ALA), lignans, and dietary fiber, and its intake lowers blood pressure in hypertensive patients. Here, we examined the effects of flaxseed powder, which includes all flaxseed components, flaxseed oil, composed mainly of ALA, flaxseed lignan, and flaxseed fiber, on hypertension and renal damage induced by deoxycorticosterone acetate (DOCA)-salt. Then, we investigated the mechanisms of action associated with the effects of flaxseed. **Methods:** Flaxseed powder, oil, lignan, or fiber was administered to DOCA-salt rats. Systolic blood pressure (SBP), urinary protein excretion, renal angiotensin converting enzyme (ACE) activity, sympathetic nerve activity, and gene expression of inflammatory mediators in the kidney and hypothalamus were measured. **Results:** Flaxseed powder and oil reduced the increases in SBP and urinary protein excretion induced by DOCA-salt treatment, whereas lignan and fiber had no effects. Flaxseed oil suppressed the increase in renal ACE activity, sympathetic nerve activity, and gene expression of renal and hypothalamic inflammatory mediators. **Conclusion:** Flaxseed has antihypertensive and renoprotective effects in DOCA-salt rats. These

effects are likely principally exerted by ALA. Furthermore, the suppression of renal ACE activity, sympathetic nerve activity, and inflammation is partly involved in the effects of flaxseed.

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Introduction

Dietary intervention has long been considered as good strategy for controlling blood pressure [1, 2]. Flaxseed was reported to decrease the increased blood pressure and renal damage in hypertensive model rats and hypertensive patients [3–5]. Therefore, dietary intake of flaxseed is considered to be a useful strategy for managing blood pressure.

Alpha-linolenic acid (ALA), lignans, and dietary fiber are abundant in flaxseed [6]. Among these components, ALA and lignans exert antihypertensive effects by inhibiting the circulating renin-angiotensin system (RAS) [7, 8]. Furthermore, dietary fiber was also reported to exhibit antihypertensive effects, although the detailed mechanisms have not been clarified [9]. However, the involvement of these individual constituents in the antihypertensive effects of flaxseed has not been elucidated and the involvement of mechanisms of action other than circulating RAS inhibitory action remains unclear.

The deoxycorticosterone acetate (DOCA)-salt hypertensive rat is one of the most common experimental models for investigating antihypertensive action. Malignant hypertension induced by DOCA-salt treatment leads to renal damage accompanied by an activation of RAS and inflammation in the kidney [10–12]. DOCA-salt treatment also triggers overactive sympathetic nervous system tone and inflammation in autonomic nerve centers [13–15]. An increased sympathetic nervous system leads to the development and maintenance of hypertension [16]. Recently, inflammation in the brain was reported to be one of the causes of sympathoexcitation in hypertension [17]. Brain inflammation followed by an overactive sympathetic nervous system may be a key element in the pathogenesis of hypertension. In addition, this model is not expected to have antihypertensive effects from the inhibition of circulating RAS [18]. Therefore, a model of DOCA-salt-induced hypertension is useful for examining circulating RAS-independent mechanisms of hypertension.

In the present study, we examined the preventive effects of flaxseed powder, which includes ALA, lignans, and dietary fiber, and flaxseed oil, composed mainly of ALA, on the increase in blood pressure and induction of renal damage in DOCA-salt rats. We also assessed the effects of flaxseed lignan and flaxseed fiber in DOCA-salt rats to elucidate the involvement of these components in the antihypertensive effects. Last, the mechanisms of action associated with the antihypertensive and renoprotective effects of flaxseed oil were investigated, focusing on renal angiotensin-converting enzyme (ACE) activity, sympathetic nerve activity, and kidney and brain inflammation.

Materials and Methods

Reagents and Animals

DOCA was purchased from Nacalai Tesque (Kyoto, Japan). Flaxseed powder, flaxseed oil, flaxseed lignan, and flaxseed fiber were kindly provided by Nippon Flour Mills Co., Ltd. (Tokyo, Japan). Male Wistar/ST rats (150–180 g, Japan SLC, Shizuoka, Japan) were housed in an environmentally controlled room with a 12-h light/dark cycle and given standard rodent chow and tap water *ad libitum*. All rats were handled in accordance with the Institutional Animal Care and Use Committee of Kyoto Pharmaceutical University following the guidelines of the National Research Council and National Institute of Health.

Experimental Procedure

Right unilateral nephrectomy was performed on rats under anesthesia with a mixture of medetomidine, midazolam, and butorphanol. After surgery, rats ($n = 6/\text{group}$) received weekly sub-

cutaneous injections of DOCA at 40 mg/kg body weight on days 0, 7, and 14 and were provided 0.5% NaCl in drinking water from day 0 to 21 to induce hypertension. Rats subjected to sham operation ($n = 4$) were used as a sham group. The volume of water intake and urine output for 24 h were measured. Urine samples were collected, and urinary protein excretion for 24 h was measured using a commercially available kit (Micro TP-Test Wako; Wako, Osaka, Japan). Systolic blood pressure (SBP) was measured by the tail-cuff method (Model UR-5000; Ueda, Nagano, Japan). At the end of the experiment, the kidney and hypothalamus were removed and stored at -80°C . Flaxseed powder was mixed with chow for a daily intake of 1.2 g (low powder group) or 2.4 g (high powder group) and administered to rats. Flaxseed oil was administered once daily at doses of 0.6 mL/day (low oil) or 1.2 mL/day (high oil) by gavage. The doses for the powder group and oil group were set such that the amount of ALA was equal. Flaxseed lignan and flaxseed fiber were mixed with chow for a daily intake of 6.6 mg or 0.26 g, respectively, and administered to rats. The doses for the lignan group and fiber group were set based on 1.2 g of flaxseed powder (low powder) containing 6.6 mg of lignan and 0.26 g of fiber. DOCA-salt hypertensive rats given the vehicle were used as a control group.

Measurement of Sympathetic Nerve Activity

Measurement of blood pressure and sympathetic nerve activity in conscious rats was performed as described previously [19, 20]. On day 21, the left femoral artery was cannulated with polyethylene tubing, and the free end of the catheter was connected to a pressure transducer (MP5100; Edwards Lifescience, Tokyo, Japan). The SBP and mean arterial pressure (MAP) were recorded using Fluclet® Jr.2 (NAGAOKA & Co., Ltd., Hyogo, Japan) under freely moving, awake conditions. Rats were next placed on a reciprocal shaker (150 rpm, TAITEC, Saitama, Japan), and the same experiment as above was conducted under shaker stress conditions. The SBP was analyzed by power spectral analysis using the wavelet method of Fluclet® Jr.2, and a power spectral density of low-frequency band (LF: 0.25–0.75 Hz) of SBP fluctuation was calculated. The power spectral density of LF-SBP ($\text{mm Hg}/\sqrt{\text{Hz}}$) was used as an index of sympathetic nerve activity.

Measurement of ACE Activity

ACE activity in the kidney was measured using a synthetic substrate, hippuryl-His-Leu, specifically designed for ACE (Peptide Institute, Osaka Japan) [11]. The kidney homogenate was centrifuged, and the supernatant was incubated for 10 min at 37°C with 5 mmol/L hippuryl-His-Leu. The reaction was terminated by the addition of 3% metaphosphoric acid, and the mixture was centrifuged. The supernatant was applied to a reversed-phase column (4.6 mm i.d. $\times$ 250 mm; Tosoh Corporation, Kanagawa, Japan) and eluted with 10 mmol/L KH_2PO_4 and CH_3OH (1:1, pH 3.0). Hippuric acid was detected by ultraviolet absorbance at 218 nm. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 mmol of hippuric acid per min.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from rat kidney and hypothalamus using Isogen reagent (Nippon Gene, Tokyo, Japan), and cDNA was synthesized from total RNA (0.5 μg) using PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan) as described previously [21, 22]. Real-time PCR was performed with SYBR Premix Ex Taq II

Table 1. List of primers used for real-time PCR

Gene	Forward (5'–3')	Reverse (5'–3')
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
IL-1 β	CAGCTTTTCGACAGTGAGGAGA	GTCGAGATGCTGCTGTGAGA
IL-6	TCTCCTCTCCGGACTTGTGAA	CTCTCCGCAAGAGACTTCCA
TNF- α	TAGCAAACCACCAAGCGGAG	TGAAATGGCAAACCGGCTGA
MCP-1	TGTTACAGTTGCTGCCTGT	GATCTCACTTGGTTCTGGTC

PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1 β , interleukin-1 beta; IL-6, interleukin-6; TNF- α , tumor necrosis factor-alpha; MCP-1, monocyte chemoattractant protein-1.

(Takara Bio Inc.) using the Thermal Cycler Dice Real-Time System (TP850, Takara Bio Inc.). The primer sequences used for real-time PCR are listed in Table 1. Data were normalized to GAPDH expression by the $\Delta\Delta C_T$ comparative method and expressed as a fold change compared with the sham group.

Statistical Analysis

All data were expressed as means $\pm$ SEM. Results were analyzed by one-way analysis of variance for repeated-measures, followed by Fisher's protected least significant difference test or one-way analysis of variance for multiple comparisons followed by the protected least significant difference test. A value of $p < 0.05$ was considered significant.

Results

Effects of Flaxseed Powder and Flaxseed Oil on Hypertension and Renal Damage in DOCA-Salt Rats

In the control group, the SBP was increased to >200 mm Hg and urinary protein excretion was increased to >300 mg/day on day 21. Low-dose and high-dose flaxseed powder both suppressed the development of hypertension from the early phase (day 7) of DOCA-salt treatment and significantly inhibited the increase in SBP as compared with the control group throughout the experimental period (Fig. 1a). Similarly, these groups had a significant decrease in urinary protein excretion as compared with the control group (Fig. 1b). Low-dose and high-dose flaxseed oil also suppressed the development of hypertension from the early phase (day 7) of DOCA-salt treatment and significantly inhibited the increase in SBP as compared with the control group throughout the experimental period (Fig. 1c). High-dose flaxseed oil significantly decreased urinary protein excretion as compared with the control group (Fig. 1d). There was no significant difference in water intake or urine output among the groups, thus salt loading was equivalent (Table 2).

Effects of Flaxseed Lignan and Flaxseed Fiber on Hypertension and Renal Damage in DOCA-Salt Rats

Weekly increases in SBP and urinary protein excretion were observed in the control group. Lignan and fiber had no effects on these parameters (Fig. 2a, b) in contrast to flaxseed powder and flaxseed oil. There was no significant difference in water intake or urine output among the groups (Table 3).

Effects of Flaxseed Oil on Renal ACE Activity in DOCA-Salt Rats

The effects of flaxseed oil on ACE activity in the kidney were examined. As shown in Figure 3, ACE activities in the control group were higher than that in the sham group. Flaxseed oil treatment significantly suppressed the activation of ACE observed in the DOCA-salt hypertensive rat kidney.

Effects of Flaxseed Oil on MAP and Sympathetic Nerve Activity in DOCA-Salt Rats

The effects of flaxseed oil on MAP and sympathetic nerve activity were first examined under free-moving, awake conditions. As shown in Figure 4a and b, flaxseed oil significantly suppressed the increases in MAP and sympathetic nerve activity in DOCA-salt rats. Next, the effects of flaxseed oil on MAP and sympathetic nerve activity were examined under shaker stress conditions. As shown in Figure 4c and d, the increases in MAP and sympathetic nerve activity induced by shaker stress in the DOCA-salt rats were reduced by flaxseed oil.

Effects of Flaxseed Oil on the Gene Expression of Inflammatory Cytokines and Chemokines in DOCA-Salt Rat Kidney and Brain

The effects of flaxseed oil on the gene expression of interleukin-1 beta (IL-1 β), IL-6, tumor necrosis factor-alpha (TNF- α), and monocyte chemoattractant protein-1

Table 2. The changes in water intake and urine volume in the control, low-powder, high-powder, low-oil, and high-oil group

Parameters	Experimental group	Day			
		0	7	14	21
Water intake, mL/day	Control	30.1±1.5	76.0±10.0	93.4±15.8	80.8±26.4
	Low powder	34.1±0.9	52.2±5.5	79.6±7.9	75.4±10.6
	High powder	31.0±2.4	64.8±8.7	106.6±11.3	96.0±15.8
	Low oil	30.6±0.7	88.4±13.7	109.2±15.7	88.6±10.9
	High oil	29.7±1.5	92.8±16.7	101.0±20.1	95.0±15.1
Urine volume, mL/day	Control	16.5±1.4	61.2±8.1	74.6±13.8	63.4±21.1
	Low powder	19.8±1.8	35.8±3.7	57.4±8.3	54.2±6.3
	High powder	18.2±0.8	48.6±6.1	74.0±11.5	79.2±12.7
	Low oil	19.8±1.3	67.6±13.4	87.0±11.8	66.8±9.5
	High oil	21.6±1.2	73.2±14.7	73.2±14.4	74.8±14.5

The values represent the mean ± SEM of data from 6 animals.

Fig. 1. Effects of flaxseed powder and flaxseed oil on the SBP and urinary protein excretion in DOCA-salt rats. **a, b** Flaxseed powder (low, 1.2 g/day; high, 2.4 g/day) supplemented diets were administered from day 0 to day 21. **c, d** Flaxseed oil (low, 0.6 mL/day; high, 1.2 mL/day) was administered once daily from day 0 to day

21 by gavage. The SBP (**a, c**) and urinary protein excretion (**b, d**) were measured at the indicated time points. Data points and bars show the mean ± SEM of data from 6 animals. * $p < 0.05$ versus the control group. SBP, systolic blood pressure.

Fig. 2. Effects of flaxseed lignan and flaxseed fiber on the SBP and urinary protein excretion in DOCA-salt rats. **a, b** Flaxseed lignan (6.6 mg/day) supplemented diet and flaxseed fiber (0.26 g/day) supplemented diet were administered from day 0 to day 21. The SBP (**a**) and urinary protein excretion (**b**) were measured at the

indicated time points. Data points and bars show the mean $\pm$ SEM of data from 6 animals. There was no significant difference between the control group and the lignan or fiber group. SBP, systolic blood pressure.

Table 3. The changes in water intake and urine volume in the control, lignan, and fiber group

Parameters	Experimental group	Day			
		0	7	14	21
Water intake, mL/day	Control	35.4 $\pm$ 2.2	91.6 $\pm$ 10.2	93.0 $\pm$ 9.2	66.0 $\pm$ 14.5
	Lignan	36.1 $\pm$ 1.4	83.7 $\pm$ 3.5	93.9 $\pm$ 8.8	67.2 $\pm$ 6.1
	Fiber	34.5 $\pm$ 2.2	69.3 $\pm$ 3.6	92.0 $\pm$ 9.9	66.4 $\pm$ 9.5
Urine volume, mL/day	Control	18.8 $\pm$ 1.7	76.8 $\pm$ 13.0	71.2 $\pm$ 9.8	76.8 $\pm$ 11.3
	Lignan	19.0 $\pm$ 1.3	63.5 $\pm$ 3.2	74.4 $\pm$ 6.2	72.6 $\pm$ 7.9
	Fiber	17.8 $\pm$ 2.4	49.9 $\pm$ 3.7	73.4 $\pm$ 8.4	78.8 $\pm$ 8.6

The values represent the mean $\pm$ SEM of data from 6 animals.

(MCP-1) in the kidney and the hypothalamus were examined. As shown in Figure 5, the control group had higher expression of IL-1 β , IL-6, TNF- α , and MCP-1 mRNA than the sham group in both regions. Flaxseed oil prevented the increased expression of these genes in the control group.

Discussion

In the present study, we confirmed that dietary flaxseed suppressed the development of hypertension in DOCA-salt rats. Flaxseed powder was reported to have antihypertensive effects at a dose of 30 g/day in a clinical trial [3]. The doses of flaxseed powder in this study are approximately 2–4 times that in the above clinical study

when converted to a human dosage. On the other hand, flaxseed powder-supplemented diets at a concentration of 10% (w/w) reduced the blood pressure in a hypertensive rat model [4]. The concentration of flaxseed powder in the chow was 5% (w/w) in the low-powder group and 10% (w/w) in the high-powder group in this study; the daily intake of chow in the present study was approximately 24 g. Therefore, the doses of flaxseed powder used in this study were reasonable for evaluating its effects on hypertension and renal damage in the rat model.

Flaxseed powder is rich in ALA, lignans, and dietary fiber, which are involved in antihypertensive action [5]. In the present study, the effects of flaxseed powder on hypertension and renal damage were almost the same as those of flaxseed oil, which is mainly composed of ALA. Moreover, flaxseed lignan and fiber had no significant ef-

Fig. 3. Effects of flaxseed oil on renal ACE activity in DOCA-salt rats on day 21. Flaxseed oil (1.2 mL/day) was administered once daily from day 0 to 21 by gavage. Rats were sacrificed on day 21, the kidney was removed, and the ACE activity in the kidney was measured. Columns and bars show the mean $\pm$ SEM of data from 4 or 6 animals. * $p < 0.05$ versus the control group. ACE, angiotensin-converting enzyme.

Fig. 4. Effects of flaxseed oil on MAP and sympathetic nerve activity in DOCA-salt rats on day 21. Flaxseed oil (1.2 mL/day) was administered once daily from day 0 to day 21 by gavage. On day 21, MAP and SBP were recorded under freely moving, awake conditions. Then, rats were placed on a reciprocal shaker, and MAP and SBP were recorded under shaker stress conditions. A power spectral density of the low-frequency band (0.25–0.75 Hz) of SBP fluctuation was calculated as an index of sympathetic nerve activity. The changes in MAP and sympathetic nerve activity were calculated by subtracting the value obtained under shaker stress from the value obtained under free-moving, awake conditions. **a** MAP under freely moving, awake conditions; **b** sympathetic nerve activity under freely moving, awake conditions; **c** MAP under shaker stress conditions; **d** sympathetic nerve activity under shaker stress conditions. Columns and bars show the mean $\pm$ SEM of data from 4 or 6 animals. * $p < 0.05$ versus the control group. MAP, mean arterial pressure.

culated by subtracting the value obtained under shaker stress from the value obtained under free-moving, awake conditions. **a** MAP under freely moving, awake conditions; **b** sympathetic nerve activity under freely moving, awake conditions; **c** MAP under shaker stress conditions; **d** sympathetic nerve activity under shaker stress conditions. Columns and bars show the mean $\pm$ SEM of data from 4 or 6 animals. * $p < 0.05$ versus the control group. MAP, mean arterial pressure.

Fig. 5. Effects of flaxseed oil on the increased gene expression of IL-1 β , IL-6, TNF- α , and MCP-1 in the kidney and the hypothalamus of DOCA-salt rats on day 21. Flaxseed oil (1.2 mL/day) was administered once daily from day 0 to day 21 by gavage. Rats were sacrificed on day 21, the kidney and the hypothalamus was removed, and the gene expression of IL-1 β (**a, e**), IL-6 (**b, f**), TNF- α

(**c, g**), and MCP-1 (**d, h**) was measured using quantitative real-time PCR. Columns and bars show the mean $\pm$ SEM of data from 4 or 6 animals. * $p < 0.05$ versus the control group. IL-1 β , interleukin-1 beta; IL-6, interleukin-6; TNF- α , tumor necrosis factor-alpha. MCP-1, monocyte chemoattractant protein-1.

fects on hypertension or renal damage. This suggests that ALA plays a central role in the antihypertensive effects of flaxseed in DOCA-salt rats. Therefore, the mechanism involved in the antihypertensive action of flaxseed oil was analyzed.

In the kidney, activation of RAS causes tissue damage through the induction of inflammation and oxidative stress [23, 24]. Inhibition of ACE prevents renal damage in DOCA-salt rats, although it does not affect the elevation of blood pressure [25]. Moreover, polyunsaturated fatty acids including ALA are reported to exhibit ACE inhibitory action and anti-inflammatory action [26, 27]. In the current study, flaxseed oil suppressed the increase in ACE activity and inflammation-related gene expression observed in the DOCA-salt rat kidney, although it had no effects on the increase in oxidative stress-related factors (data not shown). Therefore, the renoprotective effect of flaxseed seems due to the ACE inhibitory action and the anti-inflammatory action in addition to indirect action based on the antihypertensive effect.

Excessive activation of sympathetic nerve activity is a well-known cause of human hypertension. Increased sympathetic nerve activity is also involved in DOCA-salt hypertension during the early phase (1–5 days after starting treatment) and developed phase (2–6 weeks after starting treatment) [28–30]. In the present study, we demonstrated that flaxseed oil significantly suppressed the increase in sympathetic nerve activity in DOCA-salt rats. Thus, the suppression of the overactive sympathetic nervous system is thought to be one of the mechanisms of the antihypertensive effects of flaxseed. This action may play a role in the suppressive effects on the development of hypertension in the early phase of DOCA-salt treatment in addition to the effects in the developed phase. Eicosapentaenoic acid and docosahexaenoic acid, metabolites of ALA, were reported to lead to serotonin release and improve the accessibility of serotonin to its receptors [28, 31]. Released serotonin activates the 5-HT_{1B/1D} receptor expressed on sympathetic nerve terminals, decreasing noradrenalin release, thereby suppressing sympathetic neurotransmission [32, 33]. There-

fore, the modulation of serotonergic neurotransmission is likely involved in the action of flaxseed on the sympathetic nervous system. In addition, under stress conditions, increased sympathetic nerve activity and MAP were suppressed by flaxseed oil, implying that flaxseed is involved in stress tolerance. Reduction of stress is considered useful for preventing increased blood pressure because stress raises the blood pressure via sympathetic overactivity. Docosahexaenoic acid and eicosapentaenoic acid derived from ALA activate serotonergic neurotransmission and may have stress-reducing and relaxing effects.

Several studies reported that hypertension is associated with many circulating inflammatory factors [34, 35]. Increased inflammatory factors are also related to the increased risk of hypertension in normotensive individuals [36]. In recent years, inflammation in the brain has received increased attention as a cause of sympathoexcitation [37]. Hence, an inflammatory-sympathetic mechanism is a key element in the pathogenesis of hypertension. We found increased gene expression of inflammatory mediators (IL-1 β , IL-6, TNF- α , and MCP-1) in the hypothalamic region of DOCA-salt rats, as in previous reports [14, 15]. The increase in the production of proinflammatory cytokines and the infiltration of activated microglia were observed in the hypothalamus of the animal models of hypertension [37, 38]. The proinflammatory cytokines were reported to induce neuronal excitation directly [39]. Therefore, microglial activation and the subsequent production of proinflammatory cytokines may affect neuronal excitability in the hypothalamus and induce sympathoexcitation. In this study, flaxseed oil markedly suppressed the increase in proinflammatory cytokines

and chemokines observed in DOCA-salt rats. Several reports have revealed that ALA and its metabolites reduce microglial activation and the subsequent production of proinflammatory cytokines in animal models of neuroinflammation [40, 41]. Taken together, the anti-inflammatory effects of ALA and its metabolites in the brain and the subsequent suppression of sympathetic activity may play roles in the antihypertensive effects of flaxseed in DOCA-salt rats.

Conclusion

Flaxseed exhibited antihypertensive and renoprotective effects in DOCA-salt rats. Environmental stress-induced pressure was also attenuated by flaxseed. These effects were likely due to ALA. Furthermore, suppression of renal ACE activation, sympathetic nervous activation, and kidney and brain inflammation were suggested to be partially involved in the antihypertensive and renoprotective effects of flaxseed. Therefore, dietary intake of flaxseed may be a useful therapeutic strategy for the management of hypertension.

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Disclosure Statement

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