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Sesamin Metabolites Induce an Endothelial Nitric Oxide-Dependent Vasorelaxation through Their Antioxidative Property-Independent Mechanisms: Possible Involvement of the Metabolites in the Antihypertensive Effect of Sesamin

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Abbreviations: SC-1m, piperitol; SC-1, demethylpiperitol; SC-2m, (1R,2S,5R,6S)-6-(4-hydroxy-3-methoxyphenyl)-2-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3,3,0]octane; SC-2, (1R,2S,5R,6S)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane; NOS, nitric oxide synthase; NOARG, N^G-nitro-L-arginine; NO, nitric oxide; sGC, soluble guanylate cyclase; O₂⁻, superoxide; DOCA, deoxycorticosterone acetate; eNOS^{-/-}, eNOS-deficient; X/XO, xanthine/xanthine oxidase; tiron, 4,5-dihydroxy-1,3-benzene-disulfonic acid; RLU, relative light unit; Phe, phenylephrine;

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Ach, acetylcholine; ODQ, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one; PI3K, phosphatidylinositol 3-kinase; L-NAME, N^G-nitro-L-arginine-methyl ester; tempol, 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl; SBP, systolic blood pressure;

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Abstract

Sesamin, a major lignan in sesame seeds and oil, has been known to lower blood pressure in several types of experimental hypertensive animals. A recent study demonstrated that sesamin metabolites had *in vitro* radical-scavenging activities. Thus, we determined whether the antioxidative effect of sesamin metabolites modulate the vascular tone and contribute to the *in vivo* antihypertensive effect of sesamin. We used four demethylated sesamin metabolites: piperitol (SC-1m), demethylpiperitol (SC-1), (1R,2S,5R,6S)-6-(4-hydroxy-3-methoxyphenyl)-2-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3,3,0]octane (SC-2m) and (1R,2S,5R,6S)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane (SC-2). SC-1, SC-2m and SC-2, but not SC-1m, exhibited potent radical scavenging activities against the xanthine/xanthine oxidase-induced superoxide production. On the other hand, SC-1m, SC-1 and SC-2m produced endothelium-dependent vasorelaxation in phenylephrine-precontracted rat aortic rings, whereas SC-2 had no effect. The SC-1m- and SC-1-induced vasorelaxations were markedly attenuated by pretreatment with a nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine (NOARG), or a soluble guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one. Neither SC-1m nor SC-1 changed the expression level of endothelial NOS protein in aortic tissues. The antihypertensive effects of sesamin feeding were not observed in chronically NOARG-treated rats or in deoxycorticosterone acetate-salt-treated endothelial NOS-deficient mice. These findings suggest that the enhancement of endothelium-dependent vasorelaxation induced by sesamin metabolites is one of the important mechanisms of the *in vivo* antihypertensive effect of sesamin.

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Introduction

Endothelium-derived nitric oxide (NO) is a potent vasodilator. NO synthesized by endothelial NO synthase (eNOS) diffuses into the smooth muscle cells, and stimulates the soluble guanylate cyclase (sGC), leading to increased cGMP production and smooth muscle relaxation (Moncada and Higgs, 1993). Several findings have suggested that excess superoxide (O_2^-) produced in vascular cells interacts with NO, induces the abnormality of vascular tonus regulation and results in the development of hypertension (Jung et al., 2004; Rajagopalan et al., 1996). In addition, Laursen et al. (1997) reported that both the development of hypertension and the altered endothelium-dependent vascular relaxation were improved by the treatment with membrane-targeted forms of superoxide dismutase in angiotensin II-induced hypertensive rats. Thus, it seems likely that the suppression of oxidative stress improves the NO bioavailability and prevents the development of hypertension and end organ damage (Schmieder, 2005; Zhou et al., 2003).

Sesamin (Figure 1) is one of the lignans found in high concentration in sesame seeds and oil. We have obtained evidence that dietary sesamin efficiently suppressed the development and maintenance of hypertension in deoxycorticosterone acetate (DOCA)-salt-induced hypertensive (Matsumura et al., 1995), two-kidney, one-clip renal hypertensive (Kita et al., 1995), and salt-loaded stroke-prone spontaneously hypertensive rats (Matsumura et al., 1998), and that the suppressive effect of sesamin on DOCA-salt-induced hypertension was related to an inhibitory effect on the aortic O_2^- production of hypertensive animals (Nakano et al., 2003). However, the mechanisms by which sesamin-feeding reduced the O_2^- production were not clear, because sesamin itself has little radical-scavenging ability *in vitro*. A previous study investigating the metabolic pathway of sesamin, given orally to rats, demonstrated that the

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methylenedioxyphenyl moiety in the structure of sesamin was changed into a dihydroxyphenyl (catechol) moiety in the liver (Figure 1), and that the metabolic products had potent radical-scavenging activities *in vitro* (Nakai et al., 2003). Thus, we hypothesized that these radical-scavenging activities of sesamin metabolites would modulate the vascular tone and contribute to the antihypertensive effect induced by sesamin-feeding.

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Materials and Methods

Animals. Male Sprague-Dawley (SD) rats (SLC, Inc., Hamamatsu, Japan), C57bl/6J wild-type and eNOS-deficient (eNOS^{-/-}) mice (The Jackson Laboratory, Bar Harbor, ME, U.S.A.) were used. The animals were housed in an animal room under the following conditions: temperature, 24 ± 1 °C; relative humidity, 55 ± 5 %; and 12-h light-dark cycle. The animals received standard chow (NMF; Oriental Yeast, Tokyo, Japan) and drinking water *ad libitum*. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences.

Materials. Sesamin and its metabolites were prepared as described previously (Nakai et al., 2003). To obtain sesamin metabolites, sesamin was orally administered to the rat, and bile was collected through a cannula placed into the biliary duct. The sesamin metabolites (Figure 1) were obtained from this bile sample after being reacted with an enzyme mixture of glucuronidase and sulfatase. Sesamin-containing diets (1 w/w% in commercial normal diet, NMF) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). N^G-nitro-L-arginine (NOARG) and 7 α ,17 β -[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) were purchased from Peptide Institute Inc.(Osaka, Japan) and Tocris Cookson (Ballwin, MO, U.S.A.), respectively. All other reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Nacalai Tesque (Kyoto, Japan).

Measurement of Aortic O₂⁻ Production. The O₂⁻ production from xanthine/xanthine oxidase (X/XO) reaction was measured using a lucigenin-enhanced chemiluminescence assay (Nakano et al., 2003). The lucigenin concentration was set to 5 μ M, which demonstrated that its own redox cycling is minimal (Li et al., 1998; Skatchkov et al., 1999). X (200 μ M) and XO (16.2 mU/ml) were reacted in test tubes

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containing modified Krebs-HEPES buffer (pH 7.4, 99.01 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl₂, 1.20 mM MgSO₄, 1.03 mM K₂HPO₄, 25 mM Na-HEPES, 11.1 mM glucose) and incubated with or without each sesamin metabolite (50 μM) or O₂⁻ scavenger 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron; 50 μM) in the dark condition for 15 min at 37°C, before measurement. Luminescence was measured using a luminometer (Sirius-2; Funakoshi, Tokyo, Japan). Background counts were determined from identically treated xanthine-free readings and subtracted from the X/XO readings. X/XO-induced O₂⁻ production was expressed as relative light units (RLU) per min.

Isometric Tension Studies. The thoracic aortas from the male SD rats (body weight; 250-400 g) were removed, freed from fat and adherent connective tissue, and cut into strips taking special care to preserve the endothelium. In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. About 2-mm aortic segments of the thoracic aorta were suspended in organ chambers containing 10 ml of Krebs-Ringer bicarbonate solution (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 10 mM glucose) under a resting tension of 1.5 g at 37°C and gassed with 95% O₂-5% CO₂. Contractions and relaxations were measured as changes in isometric tension using a force displacement transducer (TB-612T, Nihon Kohden, Osaka, Japan) coupled to a polygraph (RM 6000, Nihon Kohden). After a 1.5-h equilibration period, vessels were contracted with 1 μM phenylephrine (Phe) and subsequently treated with 1 μM acetylcholine (Ach) to test the tissue viability. The endothelium was considered functional when the relaxation of precontracted vessels to 1 μM Ach was at least 90%. The endothelium removal was verified by the abolition or marked suppression of the Ach-induced relaxation, and by obtaining the strong relaxation (at least 90%) in response to the addition of 1 μM sodium nitroprusside. After a further equilibration period of more than 30 min was taken, the

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vessels were precontracted with 1 μ M Phe. After the precontraction reached a plateau, the relaxant response to vehicle or each sesamin metabolite (1, 10 and 50 μ M; cumulative dosage) was evaluated. Thereafter, the same vessels were equilibrated over 30 min, and used to examine the possible mechanisms of the vasorelaxant effects of sesamin metabolites. The aortic rings were pretreated with NOS inhibitor NOARG (100 μ M), sGC inhibitor 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ, 10 μ M), muscarinic receptor antagonist atropine (1 μ M), β -adrenergic receptor antagonist propranolol (10 μ M), estrogen receptor antagonist ICI 182,780 (30 μ M), histamine H₁ receptor antagonist pyrilamine (10 μ M), purine P2Y receptor antagonist reactive blue 2 (30 μ M) and phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (1 μ M), prior to the addition of Phe. All agents were treated for 15 min before precontraction with Phe. Both the concentrations and the incubation times of all agents were preliminarily ascertained to inhibit the effect of their corresponding agonist (NOARG, ODQ and atropine–ACh, 1 μ M; propranolol–isoproterenol, 1 μ M; pyrilamine–histamine, 100 μ M; reactive blue 2–ATP, 30 μ M; ICI 182,780 and wortmannin–17 β -estradiol, 30 μ M). The vasodilator response to each sesamin metabolite was expressed as a percentage of the response to Phe (1 μ M) in each tissue.

Binding assay. Competitive binding assays were performed as described previously (Sheen et al., 1985; Pruneau et al., 1998). MCF-7 human breast cancer cells were used as the source of estrogen receptor and CHO cells were used as the source of bradykinin B₂ receptor. For the assay of estrogen receptor, the homogenate of cells was centrifuged (800 \times g for 10 min), and the supernatant was collected. The cluded nuclear pellet was washed twice at 4 °C with lysis buffer, and the nuclear washes were combined with the supernatant fraction. This was centrifuged at 180,000 \times g for 30 min to yield the cytosol. Aliquots of cytosol were incubated with [³H]estradiol (1 nM) and each sesamin

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metabolite (10 μ M) for 20 h at 4 °C. For the assay of bradykinin B₂ receptor, the homogenate of cells were centrifuged at 40,000 xg for 20 min, and resuspended to collect the membrane fraction. This was incubated with [³H]bradykinin (0.2 nM) and each sesamin metabolite (10 μ M) for 45 min at 22 °C. In both assays, after removing the unbound ligand, analysis was done by scintillation counting. Nonspecific binding was determined in the presence of 6 μ M unlabelled 17 β -estradiol or 1 μ M of unlabelled bradykinin.

Western blotting. The rat thoracic aortas were obtained as described above. Aortas were immersed in test tubes containing modified Krebs-HEPES buffer (pH 7.4, 99.01 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl₂, 1.20 mM MgSO₄, 1.03 mM K₂HPO₄, 25 mM Na-HEPES, 11.1 mM glucose) and incubated with piperitol (SC-1m) or demethylpiperitol (SC-1) (1, 10, or 50 μ M) for 6 h at 37°C. Western blotting was performed as described previously (Yamashita et al., 2003). Briefly, equal amounts of protein (30 μ g) were fractionated using 7.5% SDS-polyacrylamide gels. After transfer to nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL, U.S.A.), the blots were blocked for 60 min at 4 °C with 7.5% nonfat dry milk in TBS containing 0.1% Tween20. After blocking, the blots were incubated with anti-actin and anti-eNOS polyclonal antibody (BD Biosciences, San Jose, CA, U.S.A.) at 1:1000 dilution with 7.5% nonfat dry milk in TBS containing 0.1% Tween20 overnight at 4 °C. After washing, the preparations were further incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (Zymed Laboratories, South San Francisco, CA, U.S.A.) at 1:1000 dilution with 7.5% nonfat dry milk in TBS containing 0.1% Tween20 at room temperature for 60 min. Detection was carried out using an enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) and quantified by using the NIH IMAGE for Windows. The eNOS/actin

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protein level of each sesamin metabolite-treated vessel was normalized with that of the vehicle-treated vessel.

Chronic NOS inhibition in rats. Experiments were performed on male SD rats (6 weeks old). For NO synthase inhibition, these rats were given drinking water containing NOARG at a concentration of 2.74 mM for 4 weeks. Intravenous bolus injection of NOS inhibitor (N^G -nitro-L-arginine-methyl ester, L-NAME; 3 mg/kg) did not add to the hypertensive responses in these animals, suggesting that this dose of NOARG was adequate to block the influence of NOS on blood pressure regulation. Animals were divided into three groups: i) vehicle-treated group; ii) NOARG + normal diet group; iii) NOARG + 1% sesamin-containing diet group. Treatment with the sesamin-containing diet was started at the beginning of NOARG treatment. Normotensive control animals received distilled water as vehicle throughout the study. Systolic blood pressure (SBP) was monitored using the tail cuff method and a pneumatic pulse transducer (BP-98A, Softron, Tokyo, Japan).

Deoxycorticosterone acetate (DOCA)-salt treatment in eNOS-deficient mice. C57bl/6J wild-type and eNOS^{-/-} mice (6 weeks old) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and the right kidney was removed *via* a right flank incision. After a 1-week postsurgical recovery period, the mice were separated into a sham-operated group, DOCA-salt normal diet group and DOCA-salt sesamin containing diet-fed group. The sham-operated groups were given tap water and normal diet *ad libitum*. Mice in the DOCA-salt group were treated three times weekly with DOCA suspended in corn oil, which was administered subcutaneously (75 mg/kg), and 1% NaCl was added to their tap water for drinking. Treatment with the sesamin-containing diet was started at the beginning of DOCA-salt treatment.

Measurement of plasma concentrations of sesamin and sesamin metabolites.

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Sprague Dawly rats were fed the 1% sesamin-containing diet for 4 weeks. The blood was drawn from the abdominal aorta and was centrifuged to obtain the plasma. Metabolites were extracted from plasma samples using Oasis HLB cartridges (Waters Corp., Milford, MA) and injected into an UPLC–MS/MS system (ACQUITY UPLC system; Waters Corp., Milford, MA and Quattro Micro; Waters/Micromass., Manchester, UK). Detection of the ions was performed in the multiple reaction monitoring mode, monitoring the transition (m/z) of the precursor ion to the product ion as follow; SC-2, 329.3 to 137.1: SC-2m 344.3 to 151.2: SC-1, 341.2 to 176.2 in negative ion mode: SC-1m, 374.2 to 233.2: internal standard, 369.2 to 298.2 in positive ion mode.

Statistical Analysis. All values were expressed as the mean \pm S.E.M. For statistical analysis, we used one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Differences were considered significant at $p < 0.05$.

Results

Effects of sesamin metabolites on X/XO-induced O_2^- production. SC-1, SC-2 and SC-2m, which contain the catechol moiety in their structures, at 50 μ M prominently scavenged the X/XO-induced O_2^- production (Figure 2), and their potencies were almost the same as that obtained with a standard O_2^- scavenger, tiron. On the other hand, SC-1m, which does not contain the catechol moiety, did not exhibit an effective scavenger activity.

Sesamin metabolites-induced vasorelaxation on precontracted rat aorta. Figure 3A represents the dose-response curves of vasorelaxation induced by each sesamin metabolite. The maximal vasorelaxation response induced by SC-1, SC-2m or SC-2 were very different from each other (50 μ M: SC-1, $91.3 \pm 2.7\%$; SC-2m, $38.5 \pm 6.0\%$; SC-2, $3.9 \pm 5.4\%$) in spite of similar radical scavenging activity. SC-1m, which has little antioxidative activity, induced a potent vasorelaxation (50 μ M, $90.2 \pm 2.3\%$). Figure 3B shows an original recording of SC-1m-induced vasorelaxation in phenylephrine-precontracted rat aortic rings. The relaxation induced by SC-1m occurred gradually and was sustained over 20 min. A similar time-course response was observed with SC-1 (data not shown). Vehicle treatment did not modulate the phenylephrine-induced contraction in aortic ring over the 30 min time course. Figure 3C represents the maximal vasorelaxation response induced by 50 μ M of each sesamin metabolite or tiron. Vasorelaxations induced by SC-1m, SC-1 and SC-2m were markedly attenuated by endothelium removal, although 10-20% relaxation was seen in endothelium-denuded vessels (50 μ M: $20.4 \pm 3.6\%$, $20.6 \pm 6.6\%$, and $9.2 \pm 3.0\%$, respectively).

Effects of NOARG and ODQ on SC-1m- or SC-1-induced vasorelaxation. To investigate the mechanisms of the sesamin metabolites-induced endothelium-dependent

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vasorelaxation, the possible involvement of the NOS-NO-sGC system was evaluated. Both SC-1m- and SC-1-induced relaxations were significantly inhibited by pretreatment with NOARG (50 μ M SC-1m, $18.3 \pm 4.7\%$; 50 μ M SC-1, $21.3 \pm 4.3\%$) or ODQ (50 μ M SC-1m, $18.1 \pm 3.6\%$; 50 μ M SC-1, $19.2 \pm 1.3\%$) (Figure 4). On the other hand, both the SC-1m- and SC-1-induced relaxations were unaltered by pretreatment with atropine, propranolol, ICI 182,780, pyrilamine, reactive blue 2, or wortmannin (Table 1).

Binding affinities of sesamin metabolites to the estrogen receptor. SC-1m and SC-1 (10 μ M, respectively) displaced from [3 H]17 β -estradiol (1 nM) at 11% and 54%, respectively. Both SC-2m and SC-2 (10 μ M, respectively) did not compete with the [3 H]17 β -estradiol. All metabolites had no binding affinities to the bradykinin B₂ receptor (data not shown).

Effects of sesamin metabolites on aortic eNOS protein. The possibility that sesamin metabolites regulate the expression of eNOS protein was evaluated. Western blot analysis for eNOS was performed on the isolated rat aortas incubated with vehicle, SC-1m or SC-1 for 6 h, and revealed no differences in the relative levels of eNOS expression between the vehicle- and sesamin metabolite-treated vessels (Figure 5).

Effects of the sesamin feeding on NOS inhibitor-induced hypertension in rats. The blood pressure of NOARG-treated animals markedly increased at day 2 of the experimental period (Figure 6), and the elevated SBP was sustained at a high level throughout the 4 weeks (SBP; baseline: 107.1 ± 1.9 mmHg, at 4 weeks: 146.3 ± 2.7 mmHg). Changes of SBP of NOARG-treated sesamin-fed rats (SBP; baseline: 107.47 ± 1.33 mmHg, at 4 weeks: 143.51 ± 2.85 mmHg) were almost identical to those of normal diet-fed hypertensive control rats.

Effects of sesamin feeding on DOCA-salt-induced hypertension in eNOS^{-/-} mice. In wild-type mice, DOCA-salt treatment for 5 weeks significantly increased the blood

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pressure (SBP; baseline: 102.8 ± 0.9 mmHg, at 5 weeks: 126.8 ± 2.5 mmHg) (Figure 7a), and sesamin-feeding prominently suppressed this blood pressure elevation (SBP; baseline: 103.0 ± 1.1 mmHg, at 5 weeks: 113.8 ± 2.5 mmHg). However, in eNOS^{-/-} mice, the DOCA-salt-induced increase in blood pressure (SBP; baseline: 124.7 ± 1.8 mmHg, at 5 weeks: 150.7 ± 5.5 mmHg) was not affected by 5 weeks of sesamin feeding (SBP; baseline: 124.5 ± 1.1 mmHg, at 5 weeks: 147.1 ± 2.7 mmHg) (Figure 7b).

Plasma concentrations of sesamin and sesamin metabolites. Plasma concentrations of sesamin metabolites after sesamin feeding to rats are shown in Table 2. After 4-weeks feeding of 1% sesamin-containing diet, each sesamin metabolite was detected in rat plasma at concentrations over 1 μ M, and SC-1 was a major metabolite in the plasma.

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Discussion

We previously reported that chronic sesamin-feeding effectively suppressed the DOCA-salt-induced hypertension and the enhancement of aortic O_2^- production, whereas a combination of classical antihypertensive drugs (reserpine, hydralazine and thiazide) failed to decrease the O_2^- production despite the fact that the blood-pressure lowering effect was more potent than in the case of sesamin-feeding (Nakano et al., 2003). Thus, we suggested that the antioxidative activity of sesamin-feeding contributes to its antihypertensive action. However, the mechanisms by which sesamin feeding reduced the O_2^- production were not clear, because sesamin itself has little radical-scavenging ability *in vitro* (Nakai et al., 2003). A previous study (Nakai et al., 2003) demonstrated that several metabolites, which have a mono- or di-catechol moiety in their structures and exhibit strong radical scavenging activities, were found in the bile after the oral administration of sesamin. We also observed the radical scavenging activities of sesamin metabolites, especially SC-1, SC-2m and SC-2. These findings led us to hypothesize that the radical scavenging activity of sesamin metabolites may modulate vascular tone by improving NO bioavailability. However, a potent NO-dependent vasorelaxation was observed in aorta treated with SC-1m, which has little antioxidative activity, and there was no correlation between the antioxidative activity and the vasorelaxing activity of each sesamin metabolite. Thus, it seems likely that sesamin metabolites induce the NO-dependent vascular relaxation irrespective of their antioxidant activity.

In our previous study (Nakano et al., 2003), sesamin-feeding and tempol (an SOD mimetic) treatment significantly suppressed the enhancement of vascular O_2^- production in DOCA-salt-treated rats, to the same extent. However, the degree of the suppressing effect on blood pressure elevation was greater in the sesamin fed-rats than

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in the tempol-treated rats, indicating that the antihypertensive effect of sesamin-feeding was not only due to the antioxidative effect, but also due to the other unknown mechanisms. One of these unknown mechanisms may be closely related to the sesamin metabolites-induced NO-dependent vasorelaxing effect, which is independent of their antioxidative properties. The findings that the sesamin feeding-induced antihypertensive effect was not observed in chronically NOARG-treated rats or in DOCA-salt-treated eNOS^{-/-} mice, supported this possibility.

The precise upstream mechanism by which sesamin metabolites stimulate NOS-NO-sGC signaling was not clear. Some flavonoids derived from natural compounds, such as genistein, daidzein and apigenin, have been shown to compete with 17 β -estradiol for binding to the estrogen receptor, acting as phytoestrogens (Kuiper et al., 1998). Thus, the vasorelaxant activities of SC-1m and SC-1 may be the result of the activation of the estrogen receptor, which is known to induce the enhancement of the eNOS activity via the activation of the PI3K/Akt signaling cascade (Chambliss et al., 2000; Simoncini et al., 2003) and the NO-mediated vasorelaxation (Bolego et al., 2005). Therefore, we examined whether SC-1m and SC-1 also compete with 17 β -estradiol for binding to the estrogen receptor. In fact, SC-1 had some affinities to the estrogen receptor. However, the concentration of SC-1 required to compete with 17 β -estradiol was 10,000 higher than that of 17 β -estradiol (IC₅₀; SC-1: 11.0 μ M, 17 β -estradiol: 1.1 nM). Furthermore, preincubation with ICI 182,780 and wortmannin did not modify the relaxant responses to SC-1m and SC-1. Taken together, it is unlikely that the sesamin metabolites act as phytoestrogen, at least in their vasorelaxant effects.

NOS in the endothelium is activated by signals through many G protein-coupled receptors, such as the muscarine M₃ (Lamping et al., 2004), bradykinin B₂ (Leeb-Lundberg et al., 2005), β -adrenergic (Ferro et al., 2004), histamine H₁ (Hill et al.,

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1997) and purine P2Y (Ralevic and Burnstock, 1998) receptors. In the present study, pretreatment with atropine, propranolol, pyrilamine or reactive blue 2 did not modify the vasodilator effect of SC-1m or SC-1. Moreover, we observed that there is no competition between bradykinin and sesamin metabolites for binding to the bradykinin B₂ receptor. Thus, signaling systems through the muscarinic M₃ receptor, β-adrenergic receptor, estrogen receptor, histamine H₁ receptor, purine P2Y receptor and bradykinin B₂ receptor were not involved in the upstream mechanisms of SC-1m- and SC-1-induced NO-dependent vasorelaxation. There are many other mechanisms in regulating eNOS activity, such as increasing intracellular Ca²⁺ level including the receptor-mediated Ca²⁺ influx, protein phosphorylation (Michell et al., 2002), intracellular translocation (Goetz et al., 1999) and association with other proteins (e.g. heat shock protein 90 (García-Cardena et al., 1998), caveolin-1 (Ghosh et al., 1998) and NOS interacting protein (Dedio et al., 2001), etc.). Further evaluations are needed to determine what modifications play an important role in the sesamin metabolites-induced vasorelaxation.

Sesamin metabolites produced slight vasorelaxations (10-20%) in endothelium-denuded aortas, suggesting that sesamin metabolites act directly on the smooth muscle cells to induce vasorelaxation. One potential mechanism is that sesamin metabolites may activate the neuronal NOS in smooth muscle cells. However, sesamin metabolites-induced vasorelaxations in endothelium-denuded aortas were similar to those in NOARG-treated aortas. Thus, it is difficult to consider that neuronal NOS contributes to the sesamin metabolites-induced endothelium-independent vasorelaxation. The other mechanisms, which are related to the smooth muscle relaxation, such as inhibition of Ca²⁺ influx, inhibition of phosphodiesterase or opening K⁺ channel, may contribute to these endothelium-independent vasorelaxations.

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In the present experimental condition, SC-1, SC-2m and SC-2 exhibited similarly potent radical-scavenging properties against the X/XO-induced O_2^- production, suggesting that the radical-scavenging properties of these sesamin metabolites were largely attributable to the catechol moiety in their structures. However, the catechol moiety is not critical for the vasorelaxant activity of sesamin metabolites, since the presence of the catechol moiety does not correlate to the vasorelaxant potencies of sesamin metabolites. Rather, one of the important determinants for this activity may be the presence of the methylenedioxyphenyl moiety, which is contained in SC-1m and SC-1.

The sesamin-feeding-induced antioxidative effect contributed to the lowering of blood-pressure in DOCA-salt hypertensive rats (Nakano et al., 2003). Therefore, we expected that sesamin-feeding would produce some antihypertensive effects in NOS-nonfunctional models via antioxidative mechanisms, even though the sesamin metabolites-induced NO-dependent vasorelaxant activity is an important mechanism of the sesamin-induced antihypertensive effect. However, sesamin-feeding failed to suppress the blood pressure elevation in NOARG-treated rats and DOCA-salt-treated eNOS^{-/-} mice. Recent studies demonstrated that tempol treatment had no effect on the blood pressure elevation in L-NAME-treated rats (Preti et al., 2005; Yanes et al., 2005), and that DOCA-salt treatment did not increase the O_2^- production in the aortas of eNOS^{-/-} mice (Landmesser et al., 2003). We also noted that tempol treatment had no effect on the NOARG-induced hypertension, and that the aortic O_2^- production in NOARG-treated rats was not increased compared with that in normotensive rats (unpublished observations). Collectively, these results indicate that oxidative stress was not important, at least with regard to the blood pressure elevation of these hypertensive models. Thus, it is reasonable to consider that sesamin-feeding cannot

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exhibit the antioxidative property-dependent antihypertensive effect, in NOS-nonfunctional models.

In the present study, we showed that sesamin metabolites were detected in rat plasma at concentrations over 1 μM , after 4-weeks feeding of 1% sesamin-containing diet. In particular, the plasma concentration of SC-1 was higher than those of other metabolites, and reached over 10 μM , which is able to induce the vasorelaxation in rat aortic ring. Thus, it is reasonable to consider that sesamin metabolites including SC-1 contribute to the systemic effect of sesamin-feeding.

In summary, sesamin metabolites, especially SC-1 and SC-1m, elicit endothelium-, NO- and sGC-dependent vascular relaxant responses without affecting eNOS protein expression in rat aortas. Because both SC-1m (weak antioxidant) and SC-1 (strong antioxidant) exhibited similar potencies in their vasorelaxant responses, SC-1m- and SC-1-induced vasorelaxations are probably independent of their antioxidative properties. Moreover, the blood pressure elevation in NOARG-treated rats and in DOCA-salt-treated eNOS^{-/-} mice was not suppressed by sesamin-feeding, indicating that sesamin needs functional NOS to exert its antihypertensive effect. Taken together, it is possible that one of the mechanisms of the *in vivo* antihypertensive effect induced by sesamin-feeding is closely related to the sesamin metabolites-induced NO-dependent vasorelaxant effect.

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References

- Bolego C, Cignarella A, Sanvito P, Pelosi V, Pellegatta F, Puglisi L and Pinna C (2005) The acute estrogenic dilation of rat aorta is mediated solely by selective estrogen receptor- α agonists and is abolished by estrogen deprivation. *J Pharmacol Exp Ther* **313**: 1203-1208.
- Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RGW and Shaul PW (2000) Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ Res* **87**: e44-e52.
- Dedio J, König P, Wohlfart P, Schroeder C, Kummer W and Müller-Esterl W (2001) NOSIP, a novel modulator of endothelial nitric oxide synthase activity. *Faseb J* **15**: 79-89.
- Ferro A, Coash M, Yamamoto T, Rob J, Ji Y and Queen L (2004) Nitric oxide-dependent β 2-adrenergic dilatation of rat aorta is mediated through activation of both protein kinase A and Akt. *Br J Pharmacol* **143**: 397-403.
- García-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A and Sessa WC (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **292**: 821-824.
- Ghosh S, Gachhui R, Crooks C, Wu C, Lisanti MP and Stuehr DJ (1998) Interaction between caveolins-1 and the reductase domain of endothelial nitric-oxide synthase. *J Biol Chem* **273**: 22267-22271.
- Goetz RM, Thatte HS, Prabhakar P, Cho MR, Michel T and Golan DE (1999) Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* **96**: 2788-2793.
- Hill SJ, Ganellin H, Timmerman, Schwartz JC, Shankley NP, Young JM, Schunack W,

JPET #100149

- Levi R and Haas H (1997) International union of pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* **49**: 253-278.
- Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R and Brandes RP (2004) gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation* **109**: 1795-1801.
- Kita S, Matsumura Y, Morimoto S, Akimoto K, Furuya M, Oka N and Tanaka T (1995) Antihypertensive effect of sesamin. II. Protection against two-kidney, one-clip renal hypertension and cardiovascular hypertrophy. *Biol Pharm Bull* **18**: 1283-1285.
- Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B and Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139**: 4252-4263.
- Lamping KG, Wess J, Cui Y, Nuno DW and Faraci FM (2004) Muscarinic (M) receptors in coronary circulation: gene-targeted mice define the role of M2 and M3 receptors in response to acetylcholine. *Arterioscler Thromb Vasc Biol* **24**: 1253-1258.
- Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE and Harrison DG (2005) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* **111**: 1201-1209.
- Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA and Harrison DG (1997) Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation* **95**: 588-593.
- Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ and Zuraw BL (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* **57**: 27-77.
- Li Y, Zhu H, Kuppusamy P, Rouband V, Zweier JL and Trush MA (1998) Validation of

JPET #100149

- lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem* **273**; 2015-2023.
- Matsumura Y, Kita S, Morimoto S, Akimoto K, Furuya, Oka N and Tanaka T (1995) Antihypertensive effect of sesamin I. Protection against deoxycorticosterone acetate-salt-induced hypertension and cardiovascular hypertrophy. *Biol Pharm Bull* **18**: 1016-1019.
- Matsumura Y, Kita S, Tanida Y, Taguchi Y, Morimoto S, Akimoto K and Tanaka T (1998) Antihypertensive effect of sesamin III. Protection against development and maintenance of hypertension in stroke-prone spontaneously hypertensive rats. *Biol Pharm Bull* **21**: 469-473.
- Michell BJ, Harris MB, Chen Z, Ju H, Venema VJ, Blackstone MA, Huang W, Venema RC and Kemp BE (2002) Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635. *J Biol Chem* **277**: 42344-42351.
- Moncada S and Higgs A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* **329**: 2002-2012.
- Nakai M, Harada M, Nakahara K, Akimoto K, Shibata H, Miki W and Kiso Y (2003) Novel antioxidative metabolites in rat liver with ingested sesamin. *J Agric Food Chem* **51**: 1666-1670.
- Nakano D, Itoh C, Ishii F, Kawanishi H, Takaoka M, Kiso Y, Tsuruoka N, Tanaka T and Matsumura Y (2003) Effects of sesamin on aortic oxidative stress and endothelial dysfunction in deoxycorticosterone acetate-salt hypertensive rats. *Biol Pharm Bull* **26**: 1701-1705.
- Preti SC, da Cunha V, Vassallo DV and Stefanon I (2005) The superoxide dismutase

JPET #100149

- mimetic, tempol, reduces the bioavailability of nitric oxide and does not alter L-NAME-induced hypertension in rats. *Basic Clin Pharmacol Toxicol* **97**: 29-34.
- Pruneau D, Luccarini JM, Fouchet C, Defrêne E, Franck RM, Loillier B, Duclos H, Robert C, Cremers B, Bélichard P and Paquet JL (1998) LF 16.0335, a novel potent and selective nonpeptide antagonist of the human bradykinin B₂ receptor. *Br J Pharmacol* **125**: 365-372.
- Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK and Harrison DG (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* **97**: 1916-1923.
- Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* **50**: 413-492.
- Schmieder RE (2005) Mechanisms for the clinical benefits of angiotensin II receptor blockers. *Am J Hypertens* **18**: 720-730.
- Sheen YY, Ruh TS, Mangel WF and Katzenellenbogen BS (1985) Antiestrogenic potency and binding characteristics of the triphenylethylene H1285 in MCF-7 human breast cancer cells. *Cancer Res* **45**: 4192-4199.
- Simoncini T, abkin E and Liao JK (2003) Molecular basis of cell membrane estrogen receptor interaction with phosphatidylinositol 3-kinase in endothelial cells. *Arterioscler Thromb Vasc Biol* **23**: 198-203.
- Skatchkov MP, Sperling D, Hink U, Mülsch A, Harrison DG, Sindermann I, Meinertz T and Münzel T (1999) Validation of lucigenin as a chemiluminescent probe to monitor vascular superoxide as well as basal vascular nitric oxide production. *Biochem Biophys Res Commun* **254**: 319-324.
- Yamashita J, Ogata M, Itoh M, Yamasowa H, Shimeda Y, Takaoka M and Matsumura Y

JPET #100149

(2003) Role of nitric oxide in the renal protective effects of ischemic preconditioning. *J Cardiovasc Pharmacol* **42**: 419-427.

Yanes L, Romero D, Iliescu R, Cucchiarelli VE, Fortepiani LA, Santacruz F, Bell W, Zhang H and Reckelhoff JF (2005) Systemic arterial pressure response to two weeks of tempol therapy in SHR: involvement of NO, the RAS, and oxidative stress. *Am J Physiol* **288**: R903-R908.

Zhou MS, Adam AG, Jaimes EA, Raij L (2003) In salt-sensitive hypertension, increased superoxide production is linked to functional upregulation of angiotensin II. *Hypertension* **42**: 945-951.

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Figure Legends

Figure 1

Chemical structures of sesamin and sesamin metabolites. Abbreviations of sesamin metabolites are described below: piperitol, SC-1m; demethylpiperitol, SC-1; (1R,2S,5R,6S)-6-(4-hydroxy-3-methoxyphenyl)-2-(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3,3,0]octane, SC-2m; (1R,2S,5R,6S)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane, SC-2.

Figure 2

Effects of sesamin metabolites on xanthine/xanthine oxidase-induced O_2^- production. The final concentration of vehicle (DMSO) was 0.5%. Concentrations of each drug: 50 μ M. Data are expressed as the mean \pm S.E.M. (n=3)

Figure 3

Relaxant effects of sesamin metabolites in isolated rat aortic rings. A) Original tracing of SC-1m-induced vasorelaxation. B) Concentration-response curves of sesamin metabolites-induced vasorelaxation. C) Effects of endothelial denudation on sesamin metabolites-induced vasorelaxation. Vasodilator response to each sesamin metabolite was expressed as a percentage of the response to phenylephrine (1 μ M) in each aortic ring. Data are expressed as the mean \pm S.E.M. ** $p < 0.01$ vs. vasorelaxation in endothelium-intact vessels.

Figure 4

Effects of N^G -nitro-L-arginine (100 μ M) and ODQ (10 μ M) on sesamin metabolites-induced vasorelaxation. Both drugs were pre-treated 15 min before the

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phenylephrine contraction. Data are expressed as the mean \pm S.E.M. ** $p < 0.01$ vs. vasorelaxation in untreated control vessels.

Figure 5

Effects of sesamin metabolites on the expression of eNOS protein analyzed by Western blotting. Each eNOS protein level was normalized with the eNOS/actin level of vehicle-treated vessel. Data are expressed as the mean \pm S.E.M. (n=3)

Figure 6

Effect of sesamin-feeding in chronic NOS inhibitor-induced hypertensive rats. N^G-nitro-L-arginine (2.74 mM) was dissolved in drinking water. Data are expressed as the mean \pm S.E.M. (n=6)

Figure 7

Effects of sesamin feeding on DOCA-salt-induced hypertension (A) in wild-type mice (n=9) and (B) in eNOS^{-/-} mice (n=5). Data are expressed as the mean \pm S.E.M. ** $p < 0.01$ vs. sham + normal diet group. †† $p < 0.01$ vs. DOCA + normal diet group.

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TABLE 1

Relaxation responses induced by SC-1 or SC-1m, and the responses following pretreatment with several pharmacological agents.

% relaxation	control	Atro	Prop	ICI	Pyril	RB2	Wort
n	6	3	3	3	3	3	5
SC-1m	90.2 ± 2.3	82.9 ± 4.2	88.2 ± 4.4	88.6 ± 3.1	94.1 ± 3.0	89.3 ± 2.2	90.4 ± 5.1
SC-1	91.3 ± 2.7	94.7 ± 4.0	87.1 ± 5.0	90.4 ± 2.8	91.8 ± 1.3	85.9 ± 5.7	93.6 ± 2.7

Atro, atropine (1 μ M); Prop, propranolol (10 μ M); ICI, ICI 182,780 (30 μ M); Pyril, pyrilamine (10 μ M); RB2, reactive blue 2 (30 μ M); Wort, wortmannin (1 μ M); All agents were administered for 15 min before precontraction. Vasodilator response to each sesamin metabolite (50 μ M) was expressed as a percentage of the response to phe (10^{-6} M) in each tissue. Data are expressed as the mean \pm S.E.M.

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TABLE 2

Plasma concentrations of sesamin and sesamin metabolites in 1% sesamin-containing diet-fed rats.

	SC-1m	SC-1	SC-2m	SC-2	sesamin
μM	1.1 ± 0.2	12.7 ± 0.9	1.1 ± 0.1	3.0 ± 0.1	0.2 ± 0.1

Rats were fed 1% sesamin-containing diet for 4 weeks. Data are expressed as the mean \pm S.E.M. (n=4)

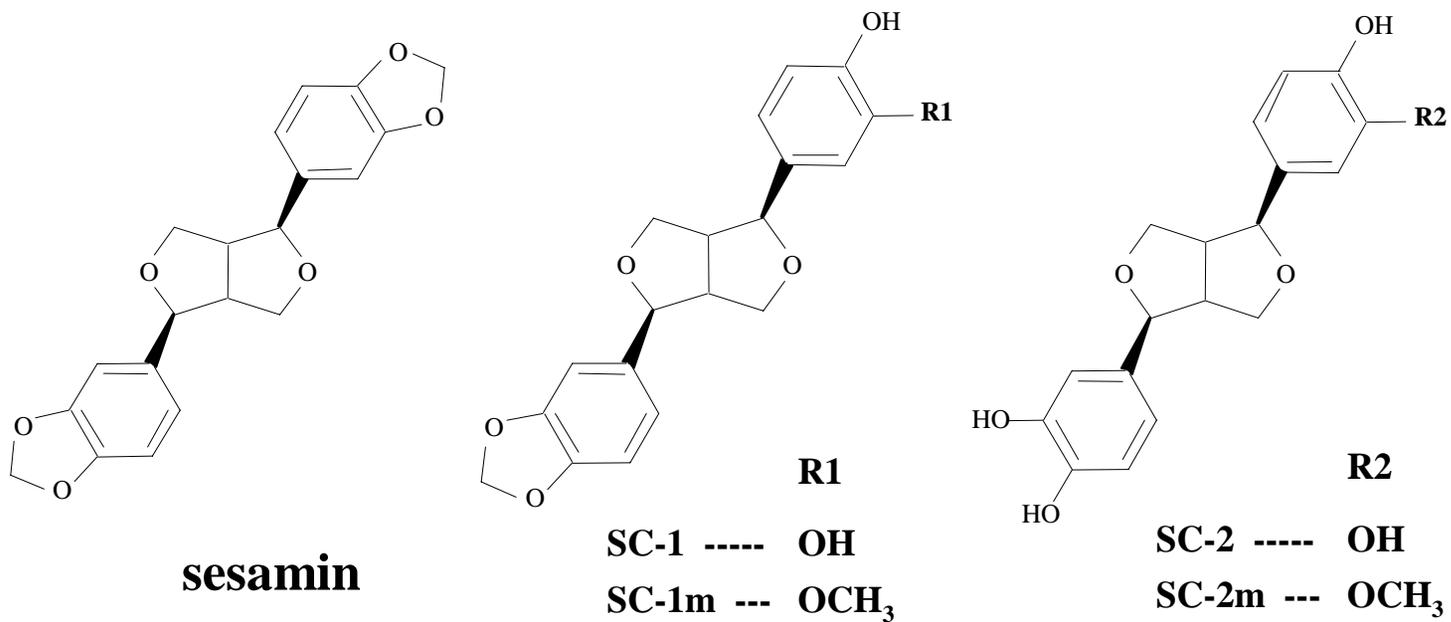


Figure 1.

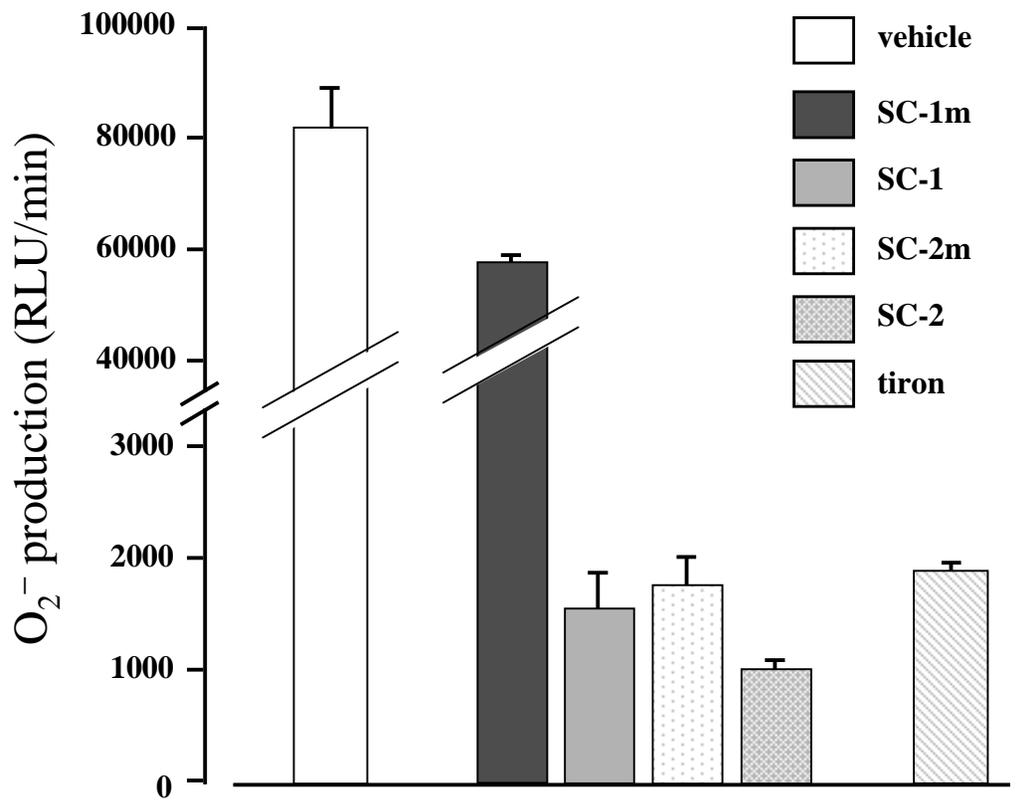


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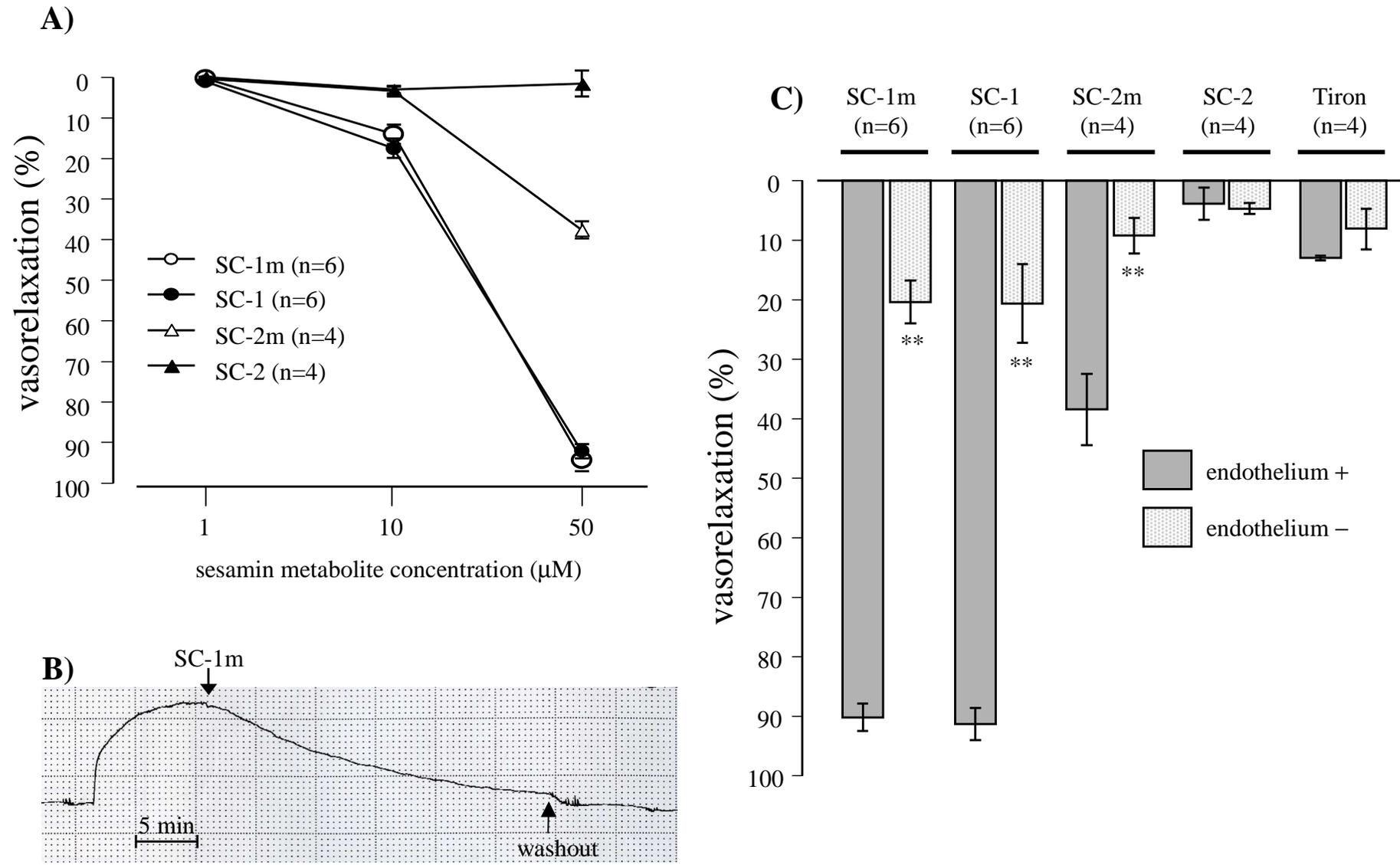


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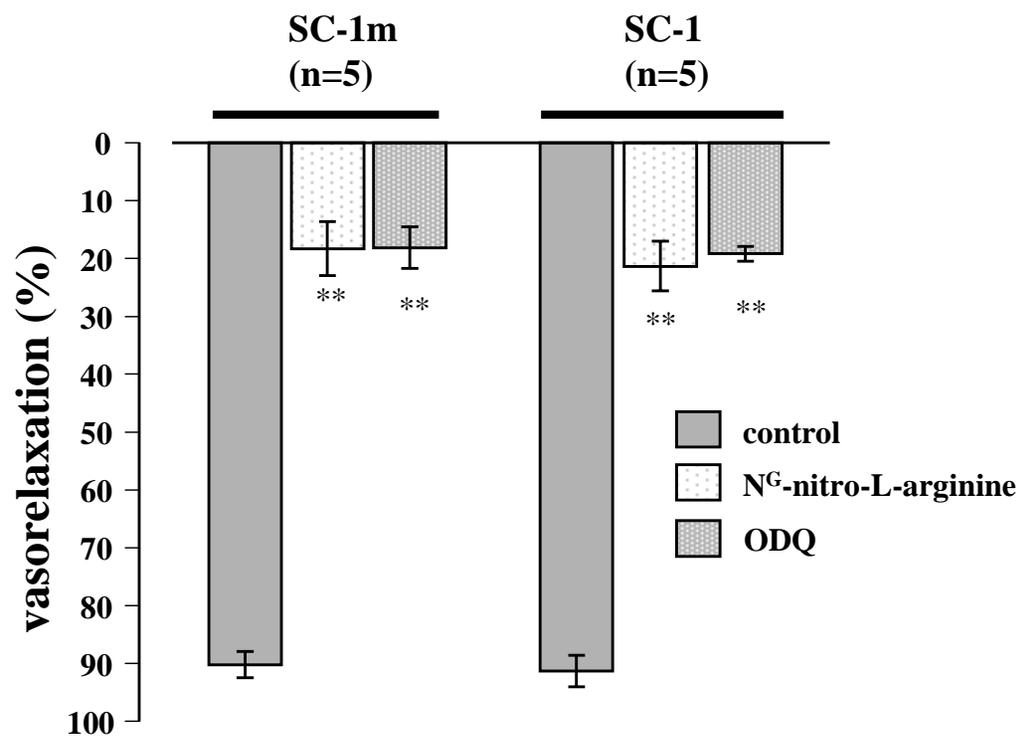


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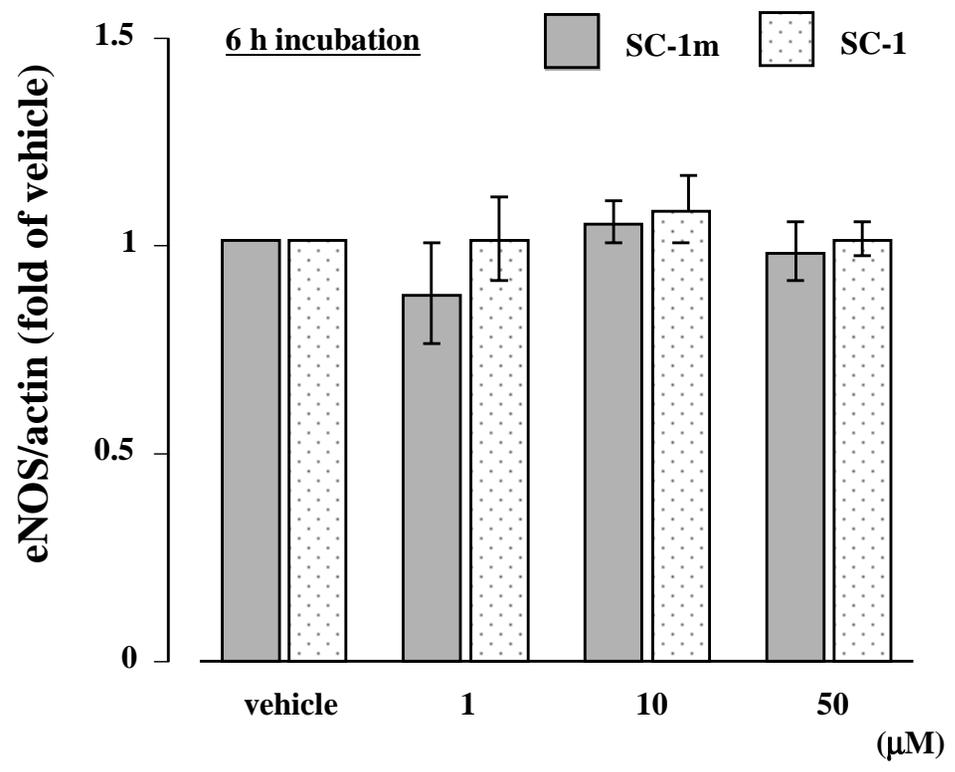


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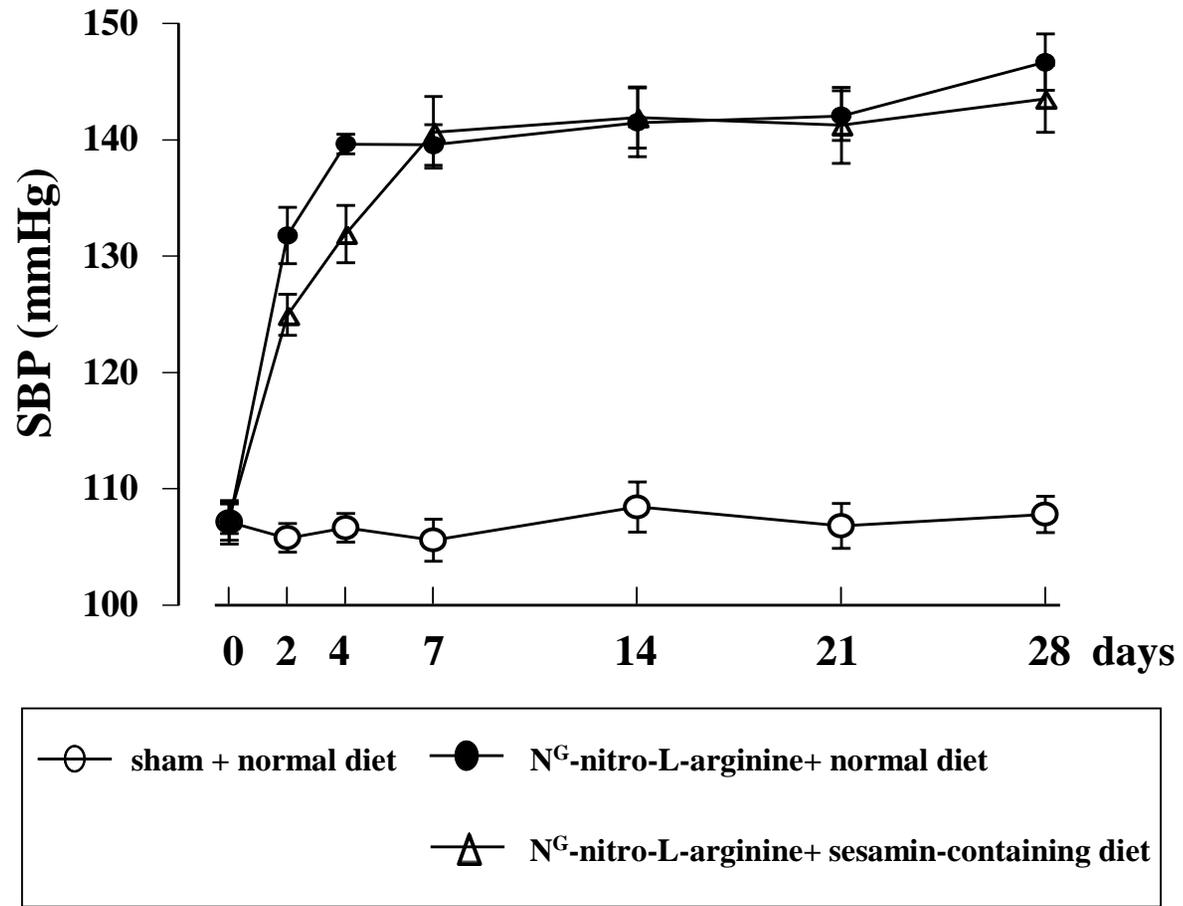


Figure 6.

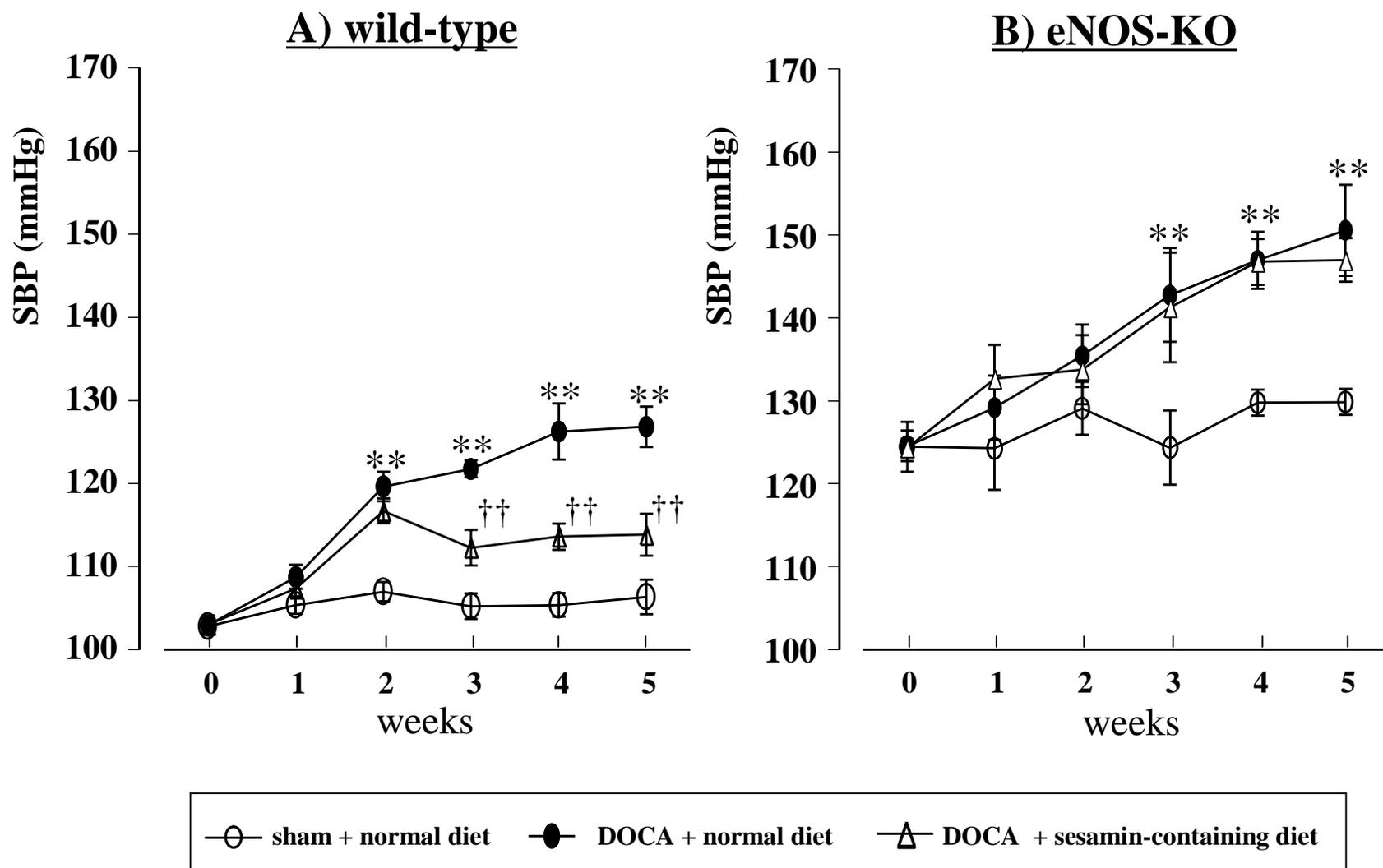


Figure 7.