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Tissue Distribution and Elimination of Estrogenic and Anti-Inflammatory Catechol Metabolites from Sesaminol Triglucoside in Rats

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Sesaminol triglucoside (STG) is the main sesame (*Sesamum indicum* L.) lignan. Like many other plant lignans, STG can be converted to the mammalian lignans by intestinal microbiota. The objectives of the present study were to investigate the distribution of STG metabolite in rats, and the effects of STG and its metabolite on in vitro inflammation and estrogenic activities. STG was metabolized via intestinal microflora to a biologically active catechol moiety which would then be absorbed into the body in rats. After oral administration of STG to Sprague−Dawley rats, the concentrations of major STG metabolites in rectum, cecum, colon, and small intestines are higher than those in liver, lung, kidney, and heart. Its concentration in brain is low but detectable. The present study demonstrates that STG may be metabolized to form the catechol metabolites first by intestinal microflora and then incorporated via intestine absorption into the cardiovascular system and transported to other tissues. Results showed that the catechol metabolites were found to be able to penetrate the tail end of intestines (large intestine) and go through urinary excretion. STG metabolites significantly reduced the production of IL-6 and TNF-α in RAW264.7 murine macrophages stimulated with lipopolysaccharide. The estrogenic activities of STG metabolites were also established by ligand-dependent transcriptional activation through estrogen receptors. This study clearly shows that STG has anti-inflammatory and estrogenic activities via metabolism of intestinal microflora.

KEYWORDS: Sesaminol triglucoside; catechol metabolites; tissue distribution; inflammation; estrogenic activity

1. INTRODUCTION

The metabolism of plant lignans involves gut microbial processes (*1*). Despite the structural diversity of plant lignans from different sources, most of them may undergo conversion to enterodiol (END) and enterolactone (ENL) by gut microbiota (*2*−*5*). The main sources of lignans in Western diets include cereals and cereal products (*6*, *7*). Plant lignans, such as matairesinol, secoisolariciresinol, pinoresinol, lariciresinol, and sesamin, are converted to enterolignans END and ENL by intestinal microbiota (*3*). A diet rich in sesame lignans has been shown to have many beneficial physiological effects, which are mostly related to its lignan compounds, such as sesaminol glucosides (STG). STG have less bioactivity in vitro, but they have been reported to be converted to bioactive phenolic compounds after oral administration (*8*−*10*).

Our previous studies reported that STG converted to mammalian lignans by intestinal microbiota involving the hydrolysis of glucoside, demethylation of a methoxy group, oxidation of dibenzylbutanediol to dibenzylbutyrolactone, and reductive cleavage of furofuran rings. STG has methylenedioxyphenyl moieties in its structure which may require additional oxidative demethylenation of the methylenedioxyphenyl ring for conversion to mammalian lignans (ST1, ST2, ST3, enterolactone, and enterodiol) (**Figure 1**). However, STG was metabolized via intestinal microflora to a biologically active catechol moiety and then absorbed into the body in rats (*11*).

There is no comprehensive information on the distribution and elimination of sesaminol triglucoside catechol metabolites (ST-2 and ST-3) in vivo. In addition, the potential bioactivities of STG metabolites are poorly understood. This study aimed to investigate the distribution of STG metabolites in vivo, and their in vitro anti-inflammatory and estrogenic activities.

2. MATERIALS AND METHODS

2.1. Chemicals. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, acetic acid, fluorescein sodium salt, and sulfatase (type H-1, from *Helix pomatia*, containing sulfatase and β-glucuronidase) were obtained from Sigma-Aldrich (Poole,

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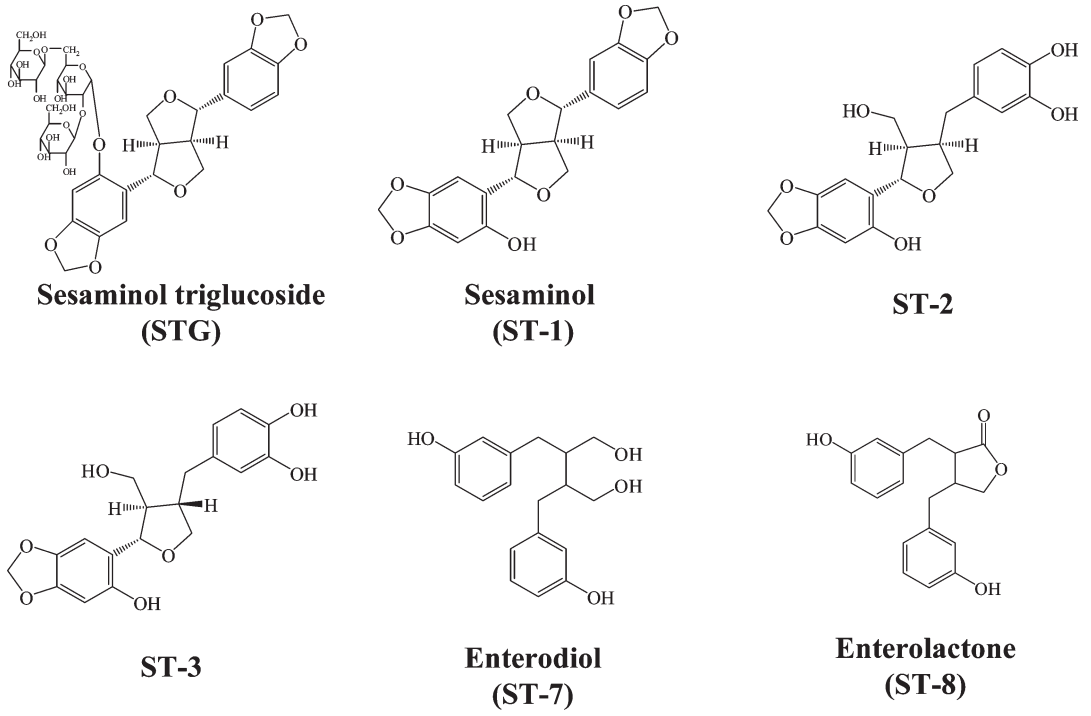

Figure 1. Chemical structures of sesaminol triglucoside and its metabolites (*11*).

Dorset, UK). The reagents lipopolysaccharide (LPS, *Escherichia coli* serotype O55: B5), pyrrolidine dithiocarbamate (PDTC), and concanavalin (Con) A (Sigma Chemical Company; St. Louis, MO, USA) were all analytical grade and dissolved in phosphate buffer saline as a stock. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and XAD-2 gel were purchased from Aldrich (Milwaukee, WI, USA). Tetramethylbenzidine (TMB) was provided by Clinical Science Products (Mansfield, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) was purchased from Fluka (St. Gallen, Switzerland). General anaerobic medium (GAM) broth was provided by Nissui (Tokyo, Japan). Antibody of IL-6 was provided by BD Pharmingen (San Diego, CA, USA). Antibody of TNF-α was provided by R&D (Minneapolis, MN, USA). All other chemicals used were of analytical grade. Liquid chromatographic grade solvents were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Triply deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Extraction and Isolation of Sesaminol Triglucoside (STG). For the isolation of STG, sesame seeds were defatted with *n*-hexane and extracted with 80% MeOH. The 80% MeOH extract was charged into an Amberlite XAD-2 column and eluted with H_2O, 20% MeOH, 40% MeOH, and 60% MeOH. The 60% MeOH fraction was then purified by preparative HPLC under the following conditions: column Cosmosil ODS (250 × 20 mm i.d.), solvent MeOH, flow rate 4 mL/min to obtain STG with 99% purity (*12*).

2.3. Preparation of Sesaminol Triglucoside (STG) Metbolites. STG metabolites were prepared according to the method of Jan et al (*11*). The incubation of STG with human intestinal bacterial mixture (300 mL of GAM broth) and isolation of STG metabolites have been described previously. Similarly, two major metabolites **ST-2**: [4-(((3*R*,4*R*)-5-(6-hydroxybenzo[*d*][1,3]dioxol-5-yl)-4-(hydroxymethyl)tetrahydrofuran-3-yl)-methyl)benzene-1,2-diol] and **ST-3**: [4-(((3S,4R,5S)-5-(6-hydroxybenzo[d]-[1,3]dioxol-5-yl)-4- (hydroxymethyl)tetrahydrofuran-3-yl)methyl)benzene-1,2-diol] were isolated and identified as described previously (*11*).

2.4. Animals and Diets. The experimental protocol was approved by the National Laboratory Animal Center (Taipei, Taiwan) Inbred male Sprague−Dawley rats [body wt 275 ± 25 g, mean ± SD] were housed in pairs in cages in a room with controlled temperature (20−22 °C), relative humidity (50−70%), and a 12-h light: dark cycle (lights on at 0700 h). The rat diet was AIN 93M diet (Purina Mills, St. Louis, MO, USA). Rats consumed their food ad libitum and had unlimited access to water; their weight and food consumption were determined weekly.

2.5. Distribution Experiment. SD rats (n = 6) were administered via gastric gavage, 500 mg/kg of body weight STG dissolved in normal saline at three daily doses (1500 mg/kg/day) for four days. After consuming the STG diet for 4 days, rats were anesthetized in the morning on the fourth day (500 mg/kg), without overnight fasting, using CO_2 as a carrier. The liver, lung, brain, plasma, small intestines, cecum, colon and rectum were collected after the administration on the fourth day. The intestines of rats were rinsed free of its contents, which would normally be a standard procedure for estimating intestine tissue concentrations. Rats were fully bled via the abdominal aorta. Blood (8−12 mL) was collected in heparin tubes and plasma was subsequently prepared in centrifuge tubes by centrifuging for 20 min at 1000*g* and 4 °C. After blood collection, the tissues were dissected, weighed, and immediately frozen in liquid nitrogen.

Preparation of Samples. All tissues were lyophilized before further processing. Rat tissues were pooled per intake group and ground and homogenized (Polytron). Liver, kidney, lung, brain, small intestines, cecum, colon and rectum required additional homogenization in the mill. Samples were stored in airtight containers at −20 °C.

Whole rat tissues were weighed in 50-mL tubes and homogenized (Polytron) in 10 mL of 0.5 mol/L sodium acetate buffer (NaOAc, pH 5.0, with 200 mg/mL ascorbic acid)/g tissue with a vortex. The samples were deproteinized with 100% acetonitrile. The extract was transferred to a 25 mL tube and centrifuged for 10 min at 10000*g* and 4 °C. The supernatant was then transferred to a clean tube and the residue was extracted two more times. The organic solvent from the supernatant was evaporated by nitrogen at 50 °C. Tubes were weighed before and after evaporation of extraction solvent. Subsequently, the residues were dissolved with 1 mL 0.1 M NaOAc buffer (pH 5.0, with 200 mg/mL ascorbic acid). Each sample (100 μL) was either hydrolyzed with 50 μL enzyme (1000 U sulfatase) in 0.1 M NaOAc buffer (pH 5.0, with 200 mg/mL ascorbic acid)/g of tissue for incubation at 37 °C, or not hydrolyzed but processed immediately with the addition of the same volume of NaOAc buffer without enzyme mix (*13*−*15*). Subsequently, all samples were deproteinized with 250 μL 100% acetonitrile and centrifuged for 10 min at 10000*g* and 4 °C. After centrifugation, 1 mL supernatant was injected into the LC-MS system. Plasma and tissues samples were analyzed by a similar method as described previously (*16*−*18*).

2.6. Excretion Experiment. Rats (n = 6) were administered via gastric gavage, 500 mg/kg body weight STG in normal saline for four days. Urine and feces samples were collected at the following time periods: 0−24 h after administration of STG. Urine and feces from rats housed in

metabolic cages were collected for the 24 h experiment. Ascorbic acid (200 mg/mL) was added to urine samples, which were then stored at −80 °C until analysis.

Preparation of Feces Samples. The feces were lyophilized before further processing. The feces were pooled per intake group and ground and homogenized (Polytron and mill). Samples were stored in airtight containers at −80 °C.

The extractions of feces in the rats were dealt with the procedures outlined in section 2.5. Urinary STG metabolites analysis was performed using a previously described method with slight modifications in rat excreta (*19*). Briefly, 2 mL of 0.1 mol/L sodium acetate buffer (pH 4.5) and 50 μL of 1000 U sulfatase activity in 0.1 mol/L sodium acetate buffer were added to 1 mL urine sample, and the mixture was incubated in a 37 °C water bath overnight to hydrolyze the conjugates of STG metabolites.

Subsequently, all samples were deproteinized by centrifuging for 10 min at 10000 × g and 4 °C. A 250 μL of 100% acetonitrile was added to each sample before deproteinization. After centrifugation, 1 mL supernatant was injected into the LC-MS system.

2.7. Determination of Sesaminol Triglucoside (STG) Metabolites in Tissues and Excreta. Determination of STG metabolites were carried out by LC-MS/MS analysis. These analyses were performed on a Thermo HPLC system equipped with electrospray-ionization ion trap mass spectrometer (ThermoFinnigan LXQ Advantage, San Jose, CA, USA). The separation was achieved using YMC Hydrosphere C18 column (2.0 × 150 mm I.D.; 5 μm, YMC, Tokyo, Japan). For the operation in MS/MS mode, a mass spectrometer with electrospray ionization (ESI) was used. During the analyses, the ESI parameters were set as follows: capillary voltage, 49 V for negative mode; source voltage, 4.5 kV; source current, 100 μA; sheath gas flow rate, 35 au; capillary temp, 350 °C; tube lens voltage, −110 V. The collision energy of m/z 359 $[M - H]^-$ was adjusted to maximize the intensity of the deprotonated molecular ion (precursor) as 30% and the collision energy was also adjusted to optimize the product ion signals as 30% for sesaminol triglucoside metabolites analysis. The selected reaction monitoring (SRM) was used to monitor the transition of the molecule to the product ion for sesaminol triglucoside metabolites analysis. All LC−MS/MS data were processed by the Xcalibur version 2.0 data acquisition software.

For the quantitation of ST2 and ST3 in the tissues and excreta, the standard curves for these two metabolites were established. The linear regression analysis showed that the correlation coefficients of all standard curves were better than 0.995 over the range of 1−100 nmol. Limits of quantification (LOQ) were calculated from the limits of detections with the LOQ being determined as five times the limit of detection (LOD). The quantification limits ranged from 1.2 nmol for ST3 to 2.1 nmol for ST2. After STG administration, the STG metabolites could be detected in the tissues and excreta.

2.8. Oxygen Radical Absorbance Capacity (ORAC). The automated ORAC assay was carried out on a Multiple-Detection Microplate Reader (Atlanta, GA, USA) with fluorescent filters (excitation wavelength, 485 nm; emission wavelength, 528 nm). In the final assay mixture (0.25 mL total volume), fluorescein (38.4 nm/L) was used as a target of free radical attack, with AAPH (3.2 mmol/L) as the $ROO^{\cdot}$ generator and sample (50 μL). Trolox (6.25−50 μmol) was used as the control standard. The analyzer was programmed to record the fluorescence of fluorescein every 5 min after addition of AAPH. Final results were calculated on the basis of the difference in the area under the fluorescein decay curve between the blank and each sample. The antioxidative activity of a sample was determined from its ability to protect the fluorescence of the indicator in the presence of peroxyl radicals. Calculations of the final ORAC values were followed by Prior et al. (*20*).

2.9. Trolox Equivalent Antioxidant Activity (TEAC). ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. For the study of STG metabolites, the $ABTS^{\bullet+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and of trolox for the standard reference data (*21*).

2.10. Transactivation Assay for Estrogenic Activity. In the transactivation assay, vectors containing GAL4-hERα (or β) ligand binding domain (LBD) chimeric receptors and the (UAS) 4-alkaline phosphatase reporter genes were cotransfected into CHO-K1 cells. Treatment of cotransfected cells with samples containing active compounds that can bind to ER LBD will then trigger the binding of GAL4 to the UAS sequence upstream of the reporter gene and activate the transcription of the reporter gene, that is, the secreted form of the human placental alkaline phosphatase (secreted alkaline phosphatase; SEAP). By measuring the alkaline phosphatase activity, the estrogen activity can then be determined. Vectors containing chimeric receptor constructs used were pBK-CMV-Gal4-hERα-ligand-binding domain (Gal4-hERαLBD) and the pBK-CMV-Gal4-hERβ- ligand-binding domain (Gal4-hERβLBD), respectively. LBD (833−1785 bp) of hERα was cloned by RT-PCR. hER cDNA was a kind gift from Dr. J. A. Gustaffson, Department of Medical Nutrition, Karolinska Institute, Huddinge. The LBD (721−1593 bp) was cloned by PCR. The LBDs of hERα and hERβ were excised and ligated to pBKCMV containing GAL4. The correct in-frame fusions were confirmed by sequencing. The reporter gene, pBK-CMV-(UAS) 4-tk-alkaline phosphatase (AP), was kindly provided by Dr. J. A. Gustaffson. The LBDs of hERα and hERβ were excised and ligated to pBKCMV containing GAL4. The correct in-frame fusions were confirmed by sequencing. These vectors were transformed into *E. coli* (XL1-Blue) and extracted by QIAGEN Plasmid Midi Kits after cultivation. CHO-K1 cells (American Type Culture Collection, Rockville, MD) were grown at 37 °C in a 5% CO_2 atmosphere in Ham's F-12 medium supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD. USA). Near confluence, cells were removed with trypsin-EDTA, seeded at a density of 2.0×10^4 cells/well into 96-well plates in the same medium supplemented with 10% of charcoal-treated FBS, and incubated for 24 h at 37 °C, 5% CO_2. Using Fugene 6 (Roche) or Lipofectamine (GIBCO) as the transfection reagent according to the manufacturer's instruction with some modification, cells were cotransfected with appropriate amounts of pBK-CMV-Gal4-hERα (or β) and pBK-CMV-(UAS) 4-tk-alkaline phosphatase (AP) in 100 μL of serum-free Ham's F-12 medium containing the appropriate amount of the transfection reagent. After 5 h, cells were treated with Ham's F-12 medium containing 10% serum replacement (TCM) (Celox, St. Paul, MN, USA) and vehicle (DMSO), 17β-estradiol (E2), or test samples (END, ENL, STG, ST1, and ST2) in appropriate concentrations. Test samples dissolved in DMSO (100 μM/100 μL) were diluted to appropriate concentrations with Ham's F-12 medium containing 10% serum replacement immediately before use. After 48 h, 50 μL of culture medium was transferred to a new 96-well plate for the reporter gene (activity of alkaline phosphatase, AP) assay. As previously described, an equal volume of SEAP assay solution containing 20 mM *p*-nitrophenyl phosphate (pNPP), 1 mM $MgCl_2$, 10 mM L-homoarginine, and 1 M diethanolamine, pH 9.8, was added and mixed (*22*, *23*). Absorbance was read at 405 nm at 15 min. Fold of activation was calculated by taking the AP activity of vehicle-treated cells as 1. Cell viability was examined using the MTS assay. The MTS assay was performed using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega Inc., Madison, WI). In brief, cells growing in the log phase were plated on 96-well plates the day before sample treatment and end point determination was performed following the manufacturer's instruction. Absorbance of the color development in sample treated and untreated cells were measured in a Bio-Rad Microplate reader. Data of transactivation obtained were considered valid only in experiments in which sample treatment did not alter cell growth and viability.

2.11. Macrophage Cell Culture Experiment. The macrophage RAW264.7 cells (Bioresource Collection and Research Center, Hsinchu, Taiwan) were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM glutamine, 1% nonessential amino acid, and 1 mM sodium pyruvate, and maintained in a humidified incubator at 37 °C in 5% CO_2. In this experiment, the RAW264.7 cells were seeded on 96-well plates at cell density of 2×10^4 cells/200 μL/well. The cells were then pretreated with various concentrations of STG metabolites for 1 h before adding 100 ng/mL LPS for stimulated incubation. STG metabolites were dissolved in DMSO for cell treatment (final DMSO concentration ≤0.1% in medium) and had no direct toxic effect on RAW264.7 cells.

2.12. Assay of Cytokine Production in RAW264.7 Cells. The production of cytokines tumor necrosis factor (TNF)-α (R&D Systems, Inc., Minneapolis, MN, USA) and interleukin (IL)-6 (PharMingen, San

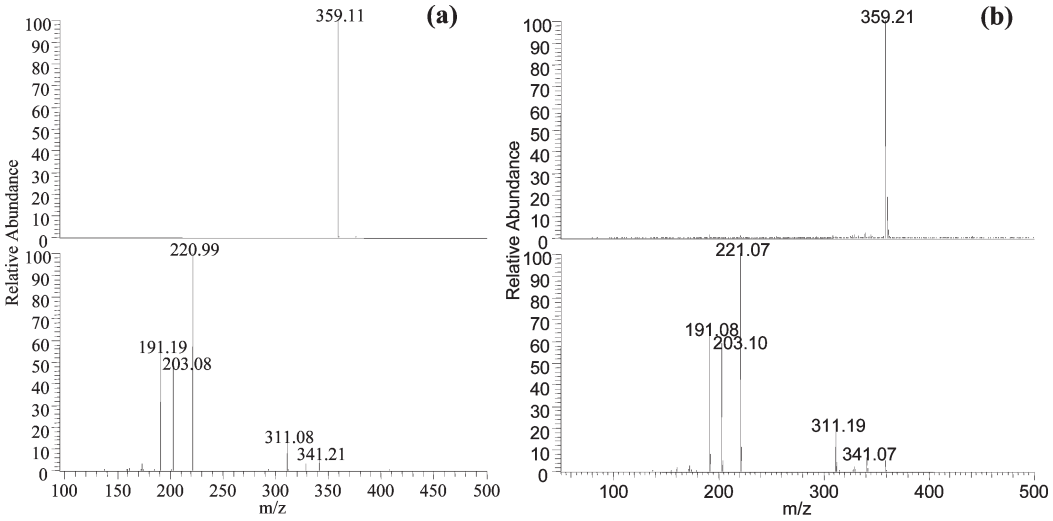

Figure 2. Mass spectra of STG metabolites in the plasma. (**a**) Mass fragmentation spectrum of MS/MS for ST-2 (*m*/*z* 359 $[M - H]^-$). (**b**) Mass fragmentation spectrum of MS/MS for ST-3 (*m*/*z* 359 $[M - H]^-$).

Diego, CA, USA) in the cell supernatants was assayed by a commercial ELISA kit. Briefly, anti-IL-6 and TNF-α antibodies were coated to 96-well plates. After incubation, the wells in the plate were washed and blocked with blocking solution (PBS buffer containing 1% bovine serum albumin; Sigma), and then washed and properly diluted supernatant or serum was added. After incubation, the wells were washed and biotin-conjugated anti-IL-6 and TNF-α antibodies were added for 2 h. The wells were then washed and horseradish peroxidase-conjugated streptavidin (Pierce Chemical Co.) was added for 30 min, washed, and incubated with ABTS or TMB (*24*). Absorbance at 405 or 620 nm was measured using ELISA reader (EL311, Bio-TEK Inc.). The data were calculated according to the cytokine standard curves.

2.13. Statistical Analysis. All samples were extracted in triplicate. Sesaminol triglucoside metabolite concentrations were expressed in μmol/g of tissue or μmol/mL plasma or urine. Tissues of six rats were pooled before analysis. Data were analyzed by *t*-test and ANOVA analysis of variance, and differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

3. RESULTS

3.1. Distribution of STG Metabolites in Rats. A full scan in negative ion mode (scan range from *m*/*z* 50 to 500) was used to identify the metabolites of STG. A tube lens offset voltage of −110 eV was applied for the determination of ST-2 and ST-3, (precursor ion is 359 $[M - H]^-$). The collision energy for collision-induced dissociation (CID) was adjusted to 30% to produce the main product ion at *m*/*z* 221, as shown in **Figure 2**. Panels **a** and **b** of **Figure 2** show the MS/MS spectra of collected rat plasma after sesaminol triglucoside administration (500 mg/kg, p.o.), with mass transitions of *m*/*z* 359 $[M - H]^- \rightarrow$ 221, 203, and 191 for ST-2 and ST-3 with sensitive selective reaction monitoring (SRM) mass spectrometry.

To investigate the distribution of enterolignans in rats, an analytical method was developed. The LC-MS/MS profile of ST-2 and ST-3 in rat urine after sesaminol triglucoside administration showed many peaks (**Figure 3a**); therefore, it is necessary to use more sensitive SRM mass spectrometry to analyze the enterolignans. **Figure 3b** shows the SRM MS/MS spectrum of the same rat urine sample as used for **Figure 3a** with mass transitions of *m*/*z* 359 $[M - H]^- \rightarrow$ 221 for ST-2 and ST-3. Two clearly separated peaks corresponding to ST-2 and ST-3 were obtained.

Tissue concentrations of ST-2 and ST-3 were analyzed by LC-MS/MS. They were widely distributed in rat tissues, with the highest concentration in plasma and the lowest in brain. ST-2 and

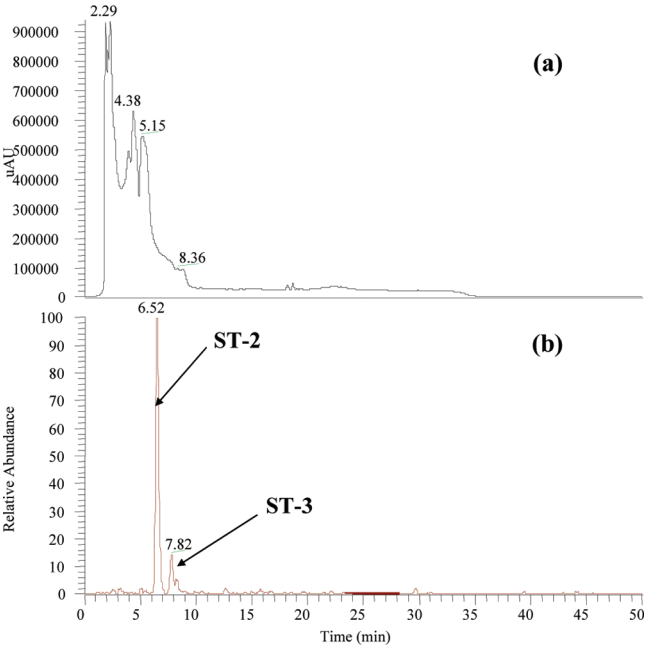

Figure 3. Mass spectrum and HPLC chromatograms of STG metabolites in the urine of rats. (**a**) A280 and (**b**) SRM for STG metabolites (*m*/*z* 359, ST-2 and ST-3).

ST-3 may be, at first, metabolized and absorbed from intestines and then transported to the other tissues (liver, lung, kidney, and brain) (**Table 1**).

It is interesting to note that, although ST-3 is the stereoisomer of ST-2, the concentrations of ST-2 were significantly higher than ST-3 in rat tissues. In the front of large intestine (cecum), the concentration ST-2 was already significantly higher than ST-3. The highest ST-2 concentrations were found in colon and cecum (**Table 2**). It is also noted that after 24 h the concentrations of ST-2 and ST-3 in the intestines (cecum, colon, and rectum) were higher than in tissues in rat (**Tables 1** and **2**). The exclusion of ST-2 and ST-3 were determined in urine and feces by LC-MS/MS within 24 h after administration of STG to rats. In urinary excretion (**Table 2**), the concentrations of ST-2 were significantly higher than ST-3. Higher concentration of ST-2 was found in the feces after administration.

Table 1. Tissue Concentration of ST-2 and ST-3 in Rat[a]

concentration (μmol/mL or μmol/g)	liver	lung	kidney	heart	plasma	brain
ST-2	0.30 ± 0.01^{c}	0.47 ± 0.02^{b}	0.29 ± 0.01^{c}	0.42 ± 0.01^{b}	36.23 ± 1.37^{a}	0.14 ± 0.01^{d}
ST-3	0.13 ± 0.01^{bc}	0.14 ± 0.01^{bc}	0.14 ± 0.01^{bc}	0.15 ± 0.01^{bc}	4.61 ± 0.39^{a}	0.12 ± 0.01^{bcd}

[a] Data are expressed as mean ± S.D. Data in the same row with different letters are significantly different at $p < 0.05$.

Table 2. Intestine Concentration of ST-2 and ST-3 in Rat[a]

concentration (μmol/mL or μmol/g)	rectum	cecum	colon	small intestines	feces	urine
ST-2	11.90 ± 0.04^{c}	20.77 ± 0.60^{a}	19.87 ± 0.16^{a}	11.90 ± 0.04^{c}	16.22 ± 0.09^{b}	6.44 ± 0.08^{d}
ST-3	3.28 ± 0.08^{b}	2.62 ± 0.06^{bc}	7.45 ± 0.87^{a}	0.189 ± 0.01^{e}	1.62 ± 0.15^{c}	0.58 ± 0.01^{d}

[a] Data are expressed as mean ± S.D. Data in the same row with different letters are significantly different at $p < 0.05$.

Table 3. Antioxidant Activities of Sesaminol Triglucoside and Its Metabolites[a]

	$ABTS^{\bullet+}$ scavenging effect	ORAC assay
sample	TEAC (μM)[b]	Trolox equivalent (μM)[c]
sesaminol triglucosdie	0.26 ± 0.06^{c}	0.77 ± 0.03^{c}
ST1 (sesaminol)	2.32 ± 0.02^{b}	7.51 ± 0.30^{b}
ST-2	6.13 ± 0.08^{a}	10.69 ± 1.30^{a}

[a] Each value represents mean ± SD from three different experiments (each experiment was conducted in triplicate). Data in the same column with different letters are significantly different at $p < 0.05$. [b] TEAC is the mM of a Trolox solution having the antioxidant capacity equivalent to a 3 μmol solution of the sample under investigation. [c] The concentration of all samples was 2 μmol.

3.2. Radical Scavenging Activity of STG and Its Metabolites in Vitro. The methylenedioxyphenyl moiety of sesaminol was changed to the 3,4-dihydroxyphenyl (catechol) moiety and aliphatic alcohol substituted tetrahydrofuran moiety in ST-2 both in vitro and in vivo (*11*). The major metabolite ST-2 was found in tissues of rats. We examined whether the metabolites had antioxidative activities in vitro in comparison with trolox. As shown in **Table 3**, the free radical scavenging activities ($ABTS^{\bullet+}$) of STG (sesaminol triglucoside), ST-1 (sesaminol), and ST-2 were 0.26 ± 0.06, 2.32 ± 0.02, and $6.13 \pm 0.08\%$, respectively. STG showed weak free radical scavenging activity, but ST-1 and ST-2 showed strong activity at 3 μmol. In the ORAC assay, STG did not show antioxidative activity, but ST-1 and ST-2 showed strong activity at 2 μmol.

3.3. Estrogenic Activity of Sesaminol Triglucoside and Its Metabolites. Endogenous estrogen, 17β-estradiol (E2), and STG metabolites all dose-dependently increased alkaline phosphatase (AP) activity in the transactivation assay (data not shown). This reporter was transfected into CHO-K1 cells, which were cultured in the absence or presence of estradiol. The AP activity was increased in the presence of estrogen. The presence and absence of estradiol in cells treated with lignans at its concentration was confirmed estrogenic activity from lignans. The potential estrogenic activity of each lignan was explored by investigating the ability of the lignans to compete with estradiol for binding to the estrogen receptor of CHO-K1 cells. If the lignan was acting as a strong estrogen, the absence of estradiol could be expected to increase its transactivation of hERα and hERβ. Additionally, the treatment of enterolignan increased the E2 cotreated activity, indicating that the sesaminol metabolites-induced transcription was ER-mediated. There were significant differences in the expression indices of hERα and hERβ treated with STG and its metabolites. The expression of hERα and hERβ were lower in cells cultured without E2 than treated with E2. The expression of hERβ was higher than hERα in the E2-maintained and -deprived cell cultures. STG showed less expression of hERα and hERβ.

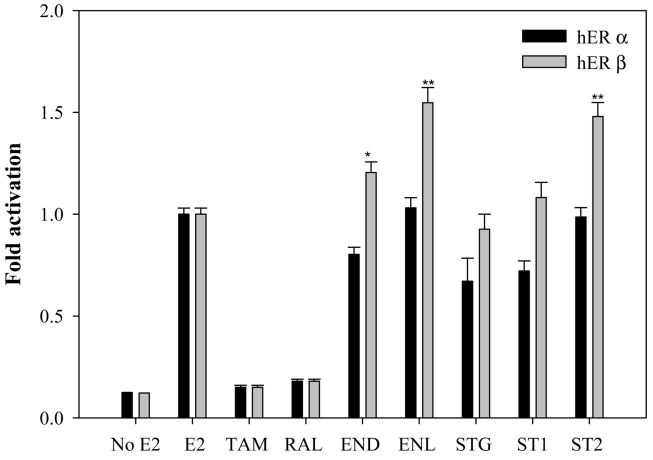

Figure 4. Transactivation of hERα and hERβ in CHO K1 cell stimulated by 0.01 nmol of E2, sesaminol triglucoside and its metabolites. E2 (17β-estradiol, 0.01 nmol) was the positive control. TAM (tamoxifen, 20 nmol) and RAL (raloxifene, 2 nmol) were the negative controls. At a similar concentration of 10 μmol, END (enterodiol), ENL (enterolactone), STG (sesaminol trigluocisde), S (sesaminol), and ST2 (the major metabolite of sesaminol triglucoside) were treated in the transactivation assay. Significant difference from the E2 group was analyzed. Values not sharing the same letter are significantly different from one another by one-way ANOVA (*$p < 0.05$ and **$p < 0.01$).

Accordingly, in cell cultures, the treatment with ST2 or its further metabolite ENL increased the hERβ expression in the presence or absence of E2. No significantly changes in hERα and hERβ expression were measured by the antiestrogens, tamoxifen (TAM) and raloxifene (RAL). However, the presence or absence of ST1 significantly affected hERβ expression in cell cultures. STG metabolites showed significant ER activity in the transactivation assay (**Figures 4** and **5**). At a similar concentration of 10 μmol, STG metabolites (ENL and ST2) showed significant activation of hERβ that was more than 1.5 fold that of 0.01 nmol of E2 (**Figure 4**) in the E2-maintained cell cultures. The metabolites (ENL, ST2) of STG activated hERβ, but the STG had no ER activity. The modest results observed with STG metabolites in the E2-maintained and -deprived cell cultures may be partially explained by the fact that STG metabolites are a partial ER agonist.

3.4. Inhibitory Activity of STG and Its Metabolites on Lipopolysaccharide-Induced Inflammation. The cells were treated with LPS alone or with various concentrations of STG and its metabolites. At concentrations used in this study, none of the LPS or STG and its metabolites treatments caused toxicity to cells

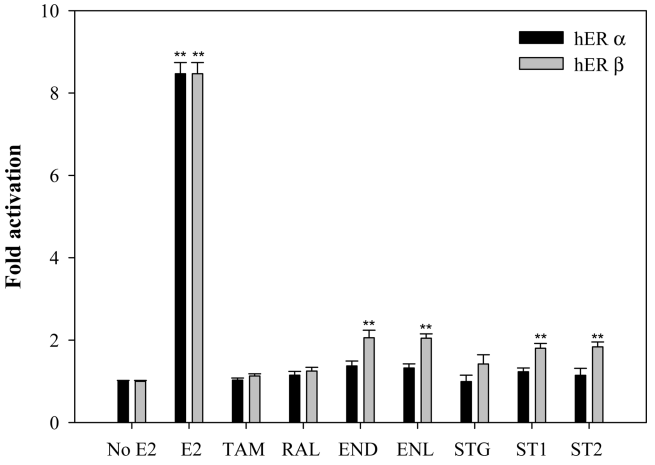

Figure 5. Transactivation of hERα and hERβ in CHO K1 cell stimulated by sesaminol triglucoside and its metabolites. E2 (17β-estradiol, 0.01 nmol) was the positive control. TAM (tamoxifen, 20 nmol) and RAL (raloxifene, 2 nmol) were the negative control. At a similar concentration of 10 μmol, END (enterodiol), ENL (enterolactone), STG (sesaminol trigluocisde), S (sesaminol), and ST2 (the major metabolite of sesaminol triglucoside) were treated in the transactivation assay. Significant difference from the No E2 group was analyzed. Values not sharing the same letter are significantly different from one another by one-way ANOVA (*p < 0.05 and **p < 0.01).

as judged by the MTS assay. The pretreatment of RAW264.7 cells with 32, 63, and 125 μg/mL STG and its metabolites, ST1 and ST2 for 1 h had no significant effect on cell viability (data not shown). As shown in **Figure 6**, STG did not suppress TNF-α secretion at a concentration of 125 μmol. On the other hand, ST1 and ST2 dose-dependently decreased LPS-induced IL-6 and TNF-α secretion from RAW 264.7 cells. ST2 (125 μmol) significantly inhibited TNF-α more than IL-6 secretion in LPS-stimulated RAW264.7 cells (0.876 and 0.004 ng/mL, respectively).

4. DISCUSSION

In a previous study, we found that STG was metabolized to enterolignans via intestinal microflora, absorbed through tail end of intestinal walls (large intestine), and then passed into the portal vein or lymphatic absorption into the cardiovascular system before it was transported to other tissues (*25*). Transit time of material through the large intestine is an important factor affecting the availability of dietary components to the host, primarily because colonic bacterial fermentation can influence circulating concentrations of compounds produced by colonic bacteria (*3*, *26*). Our previous study showed that a portion of dietary STG was absorbed and metabolized, and major portion of STG was removed in the feces after consumption (*25*). We observed only limited conversion of STG to END and ENL by in vitro fermentation with human and rat fecal microbiota; we also detected ST-2 and ST-3 in the tissues of rats after administration of STG. Therefore, it is assumed that STG is partially metabolized in the intestines to ST-2 and ST-3; they will then be further metabolized and absorbed by the intestines to hydroxylated metabolites, which are then excreted in urine. The concentrations of ST-2 in intestines were remarkably higher than those of ST-3. The concentrations of ST-2 in tissues were in the order of plasma ≫ intestines ≫ lung ≈ heart ≈ liver ≈ kidney ≈ brain. The concentrations of ST2 in both plasma and intestines were remarkably higher than END determined in a previous study . STG was metabolized to stereoisomers (ST-2 and ST-3) by intestinal microbiota. The concentration of ST-3 was extremely

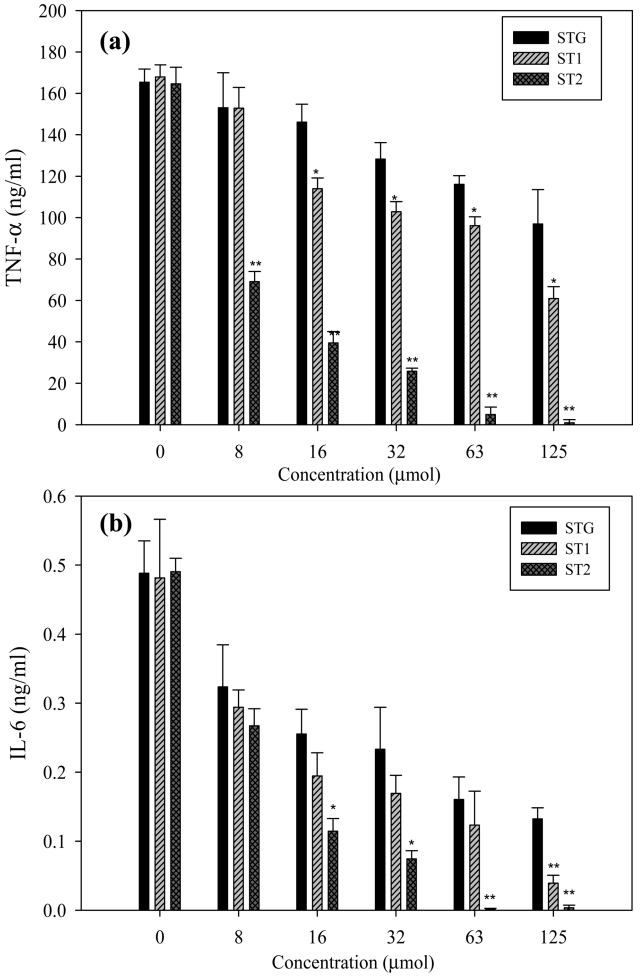

Figure 6. Effects of sesaminol triglucoside and its metabolite on (**a**) TNF-α, (**b**) IL-6 secretion from LPS-stimulated RAW264.7 cells. Values are expressed as means ± SD of three independent experiments with triplicates each and statistically analyzed by using Student's t-test. A significant difference from STG is indicated *p < 0.05 and **p < 0.01.

low compared to ST-2 in rats. ST-2 (major metabolite) had higher bioavailability compared to STG. The exact role of ST2 found in this study has to be further examined in vivo.

It was previously reported that STG has weak bioactivity in vivo, but the mechanism of this effect was unclear. Furthermore, flavonoids such as catechin, epicatechin, quercetin, kaemferol, and cyanidin generally show antioxidative effects, because they have a catechol moiety in their structures (*27*, *28*). In this study, we found that two of the metabolites of STG, ST-1, and ST-2 having a catechol moiety were novel antioxidants with much higher radical scavenging activities than STG. Previous studies noted that lignans (sesamin and sesamolin) of sesame oil regulated LPS-induced nitric oxide production in the murine microglia cell lines BV-2. Lignans significantly inhibited LPS-stimulated IL-6 mRNA and protein, and to a lesser degree TNF-α (*29*). STG exhibited a range of pharmacological activities on LPS-induced inflammatory reaction and its underlying mechanism in cultured astrocytes. STG inhibited LPS-induced generation of nitric oxide (NO) and reactive oxygen species (ROS), as well as inhibited LPS-induced cytosolic phospholipase A2 (cPLA2), cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) expression (*30*). However, following STG metabolism, we observed that ST2 had significantly higher activity than STG in inhibiting LPS stimulated TNF-α and IL-6 production in RAW 264.7 cells. The previous study demonstrated

that dietary STG inhibited the development of colonic precancerous lesions in vivo. The beneficial effect of STG might be attributed to the antioxidative property (*31*). Our previous studies reported that sesaminol triglucoside (STG) converted to ST-2 by intestinal microbiota. The concentrations of ST-2 in the intestines (cecum, colon, and plasma) were higher than other tissues in rat. Furthermore, our results of the measurement of tissues distribution, anti-inflammatory and antioxidative activities clearly demonstrated that ST-2 played a significant role.

Phytoestrogens are plant-derived compounds that can interact with estrogen receptors (ER) and exhibit estrogenic/antiestrogenic activities. Phytoestrogens can bind to ER and have a higher affinity for ERβ than for ERα (*32*). Penttinen-Damdimopoulou (*33*) reported that diet could modulate E2-induced ER-mediated responses in vivo. The dietary sources of lignans and isoflavones could modulate estrogen signaling in vivo. The expression of ERβ was significantly increased in prostate and uterus with a diet rich in sesame pericarp (30%) (*34*). According to the present results, we detected that the metabolite of STG (ST2) was endowed with estrogenic activity, which was likely to be exerted through the contribution of ER-dependent pathways. In comparison with ENL, ST2 had similar estrogenicity. This study supports the notion that dietary supplementation with STG be converted to ST2 which exhibits higher estrogenic activity than STG.

In summary, STG itself only has limited bioactivities, but when it is converted to physiologically beneficial metabolites by intestinal microflora, they may exert antioxidative, anti-inflammatory, and estrogenic activities. This further supports the potential of using dietary sesame as a disease preventive functional food.

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