# Sesamin catechol glucuronides exert anti-inflammatory effects by suppressing IFN-β and iNOS expression through the deconjugation in macrophage-like J774.1 cells

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**ABSTRACT**

Sesamin, a representative sesame lignan, has health promoting activities. Sesamin is converted into catechol derivatives and further into their glucuronides or sulfates *in vivo,* whereas the biological activities of sesamin metabolites remain unclear. We examined the inhibitory effects of sesamin metabolites on the lipopolysaccharide (LPS)-induced NO production in mouse macrophage-like J774.1 cells and found that a mono-catechol derivative SC1, (7α,7'α,8α,8'α)-3,4-dihydroxy-3',4'-methylenedioxy-7,9':7',9-diepoxylignane, has a much higher activity than sesamin and other metabolites. The inhibitory effects of SC1 glucuronides were time-dependently enhanced, associated with the intracellular accumulation of SC1 and the methylated form. SC1 glucuronides and SC1 attenuated the expression of the inducible NO synthase (iNOS) and the upstream interferon-β (IFN-β) in the LPS-stimulated macrophages. The inhibitory effects of SC1 glucuronides against NO production were cancelled by the β-glucuronidase inhibitor and enhanced by the catechol-*O*-methyltransferase inhibitor. Our results suggest that SC1 glucuronides exert the anti-inflammatory effects by inhibiting the IFN-β/iNOS signaling through the macrophage-mediated deconjugation.

**KEYWORDS:** Sesamin / Deconjugation / Macrophage / Anti-inflammation / Nitric oxide

## INTRODUCTION

Seeds and oils of sesame (*Sesamum indicum* L.) have long been recognized as traditional healthy foods in East Asian countries. Sesame seeds are a rich source of various nutrients, such as lipids, proteins, vitamins and minerals [1, 2]. In addition, the non-nutrient lignans in sesame seeds have attracted significant attention for their contribution to the biological activities and are commercially available as nutraceuticals in the form of capsules for an antioxidant function. The major sesame lignans are lipophilic sesamin and sesamolin in sesame oils and hydrophilic sesaminol glucosides in sesame seed cakes [3].

Sesamin, (7α,7'α,8α,8'α)-3,4:3',4'-bis(methylenedioxy)-7,9':7',9-diepoxylignane, has been shown to improve the lipid- and alcohol-metabolism [4-8] and have anti-oxidative [9-12], anti-inflammatory [13-15], anti-hypertensive [16-19] and anti-cancer activities [20]. Although sesamin has no anti-oxidative activity *in vitro*, it has been shown to have an anti-oxidative activity through the metabolism in liver tissue [9], indicating that sesamin metabolites may contribute to the biological effects of sesamin *in vivo*. Most of the ingested sesamin is incorporated into the liver through the portal vein, then transported to peripheral tissues through the bloodstream. A small amount of sesamin is delivered to the body through a lymphatic pathway [21]. As shown in Figure 1, in the liver, the methylenedioxyphenyl moiety of sesamin is converted into a dihydrophenyl (catechol) moiety by the drug metabolizing enzyme cytochrome P450 (CYP450) to generate the monocatechol SC1,

(7α,7'α,8α,8'α)-3,4-dihydroxy-3',4'-methylenedioxy-7,9':7',9-diepoxylignane, and dicatechol SC2, (7α,7'α,8α,8'α)-3,4:3',4'-bis(dihydroxy)-7,9':7',9-diepoxylignane. SC1 and SC2 are partially methylated by catechol-*O*-methyltransferase (COMT) to generate SC1m, (7α,7'α,8α,8'α)-3-methoxy-4-hydroxy-3',4'-methylenedioxy-7,9':7',9-diepoxylignane, and SC2m, (7α,7'α,8α,8'α)-3-methoxy-4-hydroxy-3',4'-dihydroxy-7,9':7',9-diepoxylignane. They are further conjugated by UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) to generate the corresponding glucuronides and sulfates [9, 22-25].

The catechol forms of sesamin metabolites are detected in the β-glucuronidase/sulfatase-treated human plasma, but not in the intact human plasma after the oral intake of capsules containing sesamin [24], suggesting that sesamin metabolites mostly exist as the conjugates (glucuronides and sulfates) of the sesamin catechols in the human plasma. Therefore, to understand the mechanism of action of sesamin *in vivo*, an investigation about the effects of sesamin metabolites, especially the conjugates, is essential. Moreover, sesamin catechol conjugates are expected to be deconjugated into SC1 with a higher reactivity at the inflamed sites because the neutrophils and macrophages express a β-glucuronidase-like activity especially under the inflammatory situations [26-28]. Although many studies reported the anti-inflammatory effects of sesamin *in vitro* and *in vivo* [13-15], the effects of sesamin metabolites remain to be clarified. In the present study, we investigated the anti-inflammatory activities and the mechanisms of action of

sesamin metabolites which related with deconjugation in macrophages.

## MATERIALS AND METHODS

**Chemicals and antibodies.** Sesamin and the metabolites (SC1, SC1m, SC2, SC2m, SC1-4-*O*-glucuronide (SC1-4GA), SC1-4-*O*-sulfate (SC1-4S), SC1-3-*O*-glucuronide (SC1-3GA) and SC1-3-*O*-sulfate (SC1-3S)) (Figure 1) were provided by Suntory Wellness, Ltd. (Kyoto, Japan). Sesamin and the authentic standards of sesamin metabolites (SC1, SC1m, SC2, and SC2m) were prepared using the method described previously [9, 29]. SC1-4GA, SC1-4S, SC1-3GA and SC1-3S were synthesized by referring to the methods described previously [30, 31]. One hydroxyl group of SC1 was protected with benzyl group, the other hydroxyl group was glucuronidation or sulfation. And then, SC1 conjugates were prepared by debenzylation with Pd/C under a hydrogen atmosphere. The purity of sesamin related compounds is as follows: sesamin (99.3%), SC1 (98%), SC1m (99.6%), SC2 (97%), SC2m (99%), SC1-4GA (97.6%), SC1-4S (99.8%), SC1-3GA (96.8%) and SC1-3S (99.3%). D-Saccharic acid 1,4-lactone, and antibodies against iNOS and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LPS from *Escherichia coli* O127:B8, sulfanilamide and 3,5-dinitrocatechol were purchased from Sigma-Aldrich (St. Louis, MO). *N*-1-naphthylethylenediamine dihydrochloride, phosphoric acid and sodium nitrite were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) Antibodies against the phospho-

extracellular signaling-regulated kinase 1/2 (ERK1/2) (T202/Y204), ERK1/2, phospho-c-Jun-*N*-terminal kinase (JNK) (T183/Y185), JNK, p38 mitogen-activated protein kinases (MAPK) and IκB were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The antibody against phospho-p38 MAPK (T180/Y182) was purchased from Elabscience Biotechnology, Inc. (Houston, TX). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA). The peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies were purchased from Merck Millipore (Billerica, MA). All other chemicals were purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

**Cell culture.** The mouse macrophage-like cell line J774.1 was obtained from the American Type Culture Collection (Manassas, VA). The J774.1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were grown at 37 °C under 95% humidified air and 5% $CO_2$. The passage number of J774.1 used in this study is 3-20.

**Western blot analysis.** Cells grown in a 12-well plate with 80% confluence were treated with the combination of each sesamin-related compound (25 μM) and LPS (1 μg/mL) in the FBS-free DMEM. Under the pre-treatment conditions, the cells were treated with each SC1 conjugate, then stimulated with LPS in the new medium. After treatment, the cells were washed with ice-cold phosphate buffered saline (PBS) and were lysed in a radio-immunoprecipitation assay buffer (50

mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid containing the protease inhibitor cocktail and phosphatase inhibitor cocktail (Nakalai Tesque, Inc.). After sonication and centrifugation, the supernatant was used as the protein samples. The protein concentration of the supernatant was determined using the Protein Assay Bicinchoninate Kit (Nakalai Tesque, Inc.). Equal quantities of the protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-P membrane (GE Healthcare UK, Ltd., Amersham Place, Little Chalfont, England). The membranes were blocked by EzBlock Chemi (ATTO Corporation, Tokyo, Japan) and incubated with the primary antibody overnight at 4 °C followed by the appropriate secondary antibody. The membranes were then treated with Chemi-Lumi One Super to induce the chemiluminescent signals for the detection of phosphorylated proteins and with Chemi-Lumi One L (Nakalai Tesque, Inc.) for other proteins, and the signals were detected and visualized using the Ez-Capture MG (ATTO Corporation).

**Griess assay for the quantification of nitrite.** The Griess reaction system, originally described by Griess in 1879 as a method of analysis of nitrite analysis, is based on the two-step diazotization reaction. Briefly, under acidic conditions with phosphoric acid, nitrite reacts with sulfanilamide to yield a diazonium salt. When coupled with *N*-1-naphthylethylenediamine, the diazonium salt forms a red-violet colored, water-soluble azo dye which absorbs light at 540 nm.

Cells grown in a 24-well plate with 80% confluence were treated with the combination of sesamin-related compounds (10 or 25 μM) and LPS (1 μg/mL) in the phenol red-free and FBS-free DMEM. Under the pre-treatment conditions, the cells were treated with each SC1 conjugate, then stimulated with LPS in the new medium. In the experiments, a β-glucuronidase inhibitor D-saccharic acid 1,4-lactone (2 mM) was added to the medium every 8 h to sustain the activity of the unstable D-saccharic acid 1,4-lactone. After incubation, 100 μL of the cell culture supernatant was placed in a 96-well plate and mixed with 50 μL of 1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% *N*-1-naphthylethylenediamine. The absorbance at 550 nm was measured by a microplate reader (MTP-800Lab, Hitachi High-Tech Science Corporation, Tokyo, Japan). The nitrite concentration was determined by comparison with the standard curve of sodium nitrite.

**Analysis for cellular accumulation of sesamin metabolites.** Cells grown in a 60-mm dish with 80% confluence were treated with SC1 conjugates for 0, 8, 12 and 24 h in FBS-free medium. The experiments using β-glucuronidase inhibitor were carried out in the same way as already mentioned. After incubation, the cells were washed five times with PBS and collected by scraping in a certain amount of methanol. After sonication, an aliquot of the mixture was dissolved in the solution of Protein Assay Bicinchoninate Kit to measure the protein level. The remaining was centrifuged at 15000 rpm for 5 min and the supernatant was collected, evaporated, and dissolved in 100 μL of 50% methanol. Ten μL of the sample was injected into a high-performance

liquid chromatography-electrochemical detection (HPLC-ECD) system as described below.

**HPLC-ECD.** Samples were injected into an HPLC-ECD system (ESA, Cambridge, MA) equipped with a COSMOSIL $5C_{18}$-AR-II (4.6 ID × 150 mm, Nakalai Tesque, Inc.). The compound separation was performed using the gradient elution method. Solvent A was 0.5% phosphoric acid, and solvent B was 100% acetonitrile. The gradient program was as follows: 0-2 min, 25%B; 2-20 min, linear gradient to 80%B; 20-21 min, linear gradient to 100%B; 21-25 min, hold; 25-25.1 min, 100-25%B; flow rate, 0.8 mL/min. Electrochemical detection was carried out by the coulometric electrode array system with four electrodes in the array whose potentials were incrementally set at 200, 300, 400 and 500 mV. Quantification of sesamin metabolites was performed based on the peak areas of authentic compounds (SC1, SC1m and SC1 conjugates, Supporting Information Figure S1).

**RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR).** After washing the cells with ice-cold PBS, the total cellular RNA was isolated using sepazol-RNA I super G (Nakalai Tesque, Inc.) according to the manufacturer's instructions. The RNA concentration was determined using the QuantiFluor® RNA System on a Quantus™ Fluorometer (Promega Corporation, Madison, WI). After reverse transcription into cDNA with a ReverTra Ace® qPCR RT Master Mix and gDNA remover (TOYOBO CO., LTD., Osaka, Japan), qPCR was then performed using a LightCycler® Nano real-time PCR system (Roche Diagnostics

143 K.K., Tokyo, Japan) with the THUNDERBIRD® SYBR® qPCR Mix (TOYOBO CO., LTD.) and
144 gene-specific primers. The primers used were as follows: IFN-β, 5'-
145 AGCTCCAAGAAAGGACGAACAT-3' (forward) and 5'-GCCCTGTAGGTGAGGTTGATCT-3'
146 (reverse); GAPDH, 5'-TCAAGCTCATTTCCTGGTAT-3' (forward) and 5'-
147 GTCCAGGGTTTCTTACTCCT-3' (reverse). Fold changes of the target gene (IFN-β) expression
148 were calculated by the comparative Ct method and normalized to the housekeeping gene (GAPDH)
149 expression.

150 **Statistical analysis.** The statistical significance was analyzed by a one-way analysis of
151 variance (ANOVA) followed by multiple comparisons among the means (Tukey's HSD) using R
152 software, version 3.4.2. The *P* values of $<0.05$ were regarded to be statistically significant.

153

154 **RESULTS**

155 **Sesamin metabolites exert an anti-inflammatory effect in J774.1 cells.** To check the
156 anti-inflammatory activity of sesamin and the metabolites (SC1, SC1m, SC2, SC2m, SC1-4GA,
157 SC1-4S, SC1-3GA and SC1-3S, Figure 1), we evaluated the inhibitory effects of these compounds
158 on the NO production in LPS-stimulated J774.1 cells. Upon co-treatment with each compound and
159 LPS (Figure 2), sesamin weakly inhibited LPS-increased the NO production while SC1 strongly
160 inhibited. The inhibitory effects of SC1 were attenuated by the methylation, suggesting that the

161 catechol group of SC1 seems to contribute to the inhibitory effects. The inhibitory effects of SC2
162 (dicatechol form) and the methylated form, SC2m, were weaker than that of SC1 (monocatechol
163 form). On the other hand, SC1 glucuronides (SC1-4GA and SC1-3GA) slightly inhibited the LPS-
164 induced NO production, while SC1 sulfates (SC1-4S and SC1-3S) had no effect.

165 **SC1 conjugates are time-dependently deconjugated to accumulate in macrophages.**
166 We previously revealed that a glucuronide of quercetin, a catechol-type flavonoid, is deconjugated
167 by the interaction with macrophages, resulting in the cellular accumulation of quercetin and the
168 methylated derivative [32]. Therefore, we examined the intracellular accumulation of SC1 and SC1m
169 during the treatment of macrophages with SC1 conjugates. The analysis using HPLC-ECD showed
170 the accumulation of SC1 and especially SC1m in macrophages treated with SC1 glucuronides in a
171 time-dependent manner (Figure 3A and Supporting Information Figure S2). On the other hand,
172 SC1 and SC1m were scarcely detected by the treatment with SC1 sulfates, although SC1 sulfates
173 themselves were detected. These results indicate that SC1 glucuronides are deconjugated to
174 accumulate mainly as SC1m in the macrophages and, in contrast, SC1 sulfates are only slightly
175 deconjugated.

176 Since the time-dependent accumulation of SC1 and SC1m was observed in macrophages
177 treated with SC1 conjugates, we examined whether or not the pre-treatment of SC1 conjugates
178 facilitates their anti-inflammatory activities. As shown in Figure 3B, the inhibitory effects of SC1

conjugates against the LPS-induced NO production were enhanced by the pre-treatment for 12-24 h when compared to the co-treatment conditions. The inhibitory potency of SC1-3GA was higher than SC1-4GA and SC1 sulfates scarcely have inhibitory effects, suggesting the correlation between the anti-inflammatory activities and susceptibility to deconjugation of SC1 conjugates.

**SC1 and SC1 glucuronides inhibit IFN-β and iNOS expression in LPS-stimulated macrophages.** When LPS, one of the inflammatory stimuli, is recognized by the Toll-like receptor 4 (TLR4) in macrophages, the multiple signaling pathways are activated, including the MAPK pathway, nuclear factor-κB (NF-κB) pathway and IFN-β pathway [33]. These pathways up-regulate the expression of iNOS to generate the pro-inflammatory mediator NO [34, 35]. To elucidate the molecular mechanisms of the anti-inflammatory effects of SC1 and SC1 conjugates, we investigated the involvement of these signaling pathways. The co-treatment with SC1 remarkably suppressed the LPS-induced protein expression of iNOS (Figure 4A, left), while the pre-treatment with SC1 glucuronides, but not SC1 sulfates suppressed the iNOS expression. In contrast, the inhibitory effects of the co-treatment with SC1m were not observed (Figure 4A, right). The co-treatment with SC1 slightly suppressed the phosphorylation of p38, but not of ERK and JNK, and had no effects on the degradation of IκB in the LPS-stimulated macrophages (Figure 4B). On the other hand, the pre-treatment with SC1 conjugates had little effects on the phosphorylation of ERK, JNK and p38 and the degradation of IκB in the LPS-stimulated macrophages (Figure 4C). The

LPS-induced IFN-β mRNA expression was significantly suppressed by the co-treatment with SC1 and pre-treatment with SC1 glucuronides (Figure 4D). These results suggest that the inhibitory effects of SC1 and the glucuronides on the NO production in the LPS-stimulated macrophages are mediated by the attenuation of the IFN-β transcription and iNOS protein expression.

**The anti-inflammatory effects of SC1 glucuronides are mediated by the β-glucuronidase activity of the macrophages.** Since the macrophage seems to be mainly involved in the deconjugation of the glucuronides, we examined the effect of the β-glucuronidase inhibitor, D-saccharic acid 1,4-lactone, on the cellular accumulation of SC1 and SC1m by treatment of the macrophages with SC1 conjugates. As shown in Figure 5A and Supporting Information Figure S3, the treatment of the β-glucuronidase inhibitor dramatically suppressed the cellular accumulation of SC1 and SC1m in the cells treated with SC1 glucuronides but not affected in the cells treated with SC1 sulfates. In the preliminary experiment, the supernatant of FBS-free antibiotics containing medium after the incubation with macrophages for 24 h or the fresh FBS-free antibiotics containing medium is adjusted to acidic condition (pH5.2) suitable for enzyme reaction by sodium acetate, and then incubated with SC1 glucuronides for 1 h at 37 °C in a cell-free system. As the result, SC1 conjugates were deconjugated by the incubation with the cell culture supernatant of the macrophages, but not with fresh medium (data not shown), suggesting the contribution of the secretion with β-glucuronidase activity from macrophages to the deconjugation

of SC1 glucuronides. These results indicate that the β-glucuronidase activity of the macrophage is essential for the accumulation of SC1 and SC1m in the cells treated with SC1 glucuronides.

Next, we examined the role of the β-glucuronidase activity in the anti-inflammatory effects of SC1 conjugates. The β-glucuronidase inhibitor completely impaired the inhibitory effects of SC1 glucuronides on the LPS-induced NO production (Figure 5B), suggesting that SC1 glucuronides elicit the anti-inflammatory effects through the deconjugation by the β-glucuronidase activity of the macrophage.

**The cellular accumulation of SC1m induced by the treatment of SC1 glucuronides is mediated by the COMT activity of the macrophages.** SC1 glucuronides are metabolized to accumulate mainly as SC1m in the macrophages, suggesting the involvement of COMT as well as the β-glucuronidase activity of the macrophages. Thus, we examined the effect of the COMT inhibitor, 3,5-dinitrocatechol, on the accumulation of SC1m and SC1 in the macrophages treated with SC1 conjugates. The cellular accumulation of SC1m was counteracted by the co-treatment of the COMT inhibitor accompanied with the increase in SC1 cellular accumulation (Supporting Information Figure S4). Since the anti-inflammatory effect of SC1 was stronger than that of SC1m (Figure 2), we expected that the inhibition of the COMT activity facilitates the anti-inflammatory activity of SC1 glucuronides. As expected, the inhibitory effects of SC1 glucuronides are significantly enhanced by the co-treatment of the COMT inhibitor and the effects are comparable

to SC1 (Figures 5C and 2). These results indicate that during the macrophage-mediated metabolic conversion of SC1 glucuronides into SC1 and SC1m, SC1 could act as the active form for the anti-inflammatory effects.

## DISCUSSION

Sesamin, one of the major sesame lignans, is known to have numerous physiological effects related to health promotion. Since sesamin has an anti-oxidative activity *in vivo* but not *in vitro* [9], sesamin metabolites are expected to play important roles in the biological effects of sesamin. Orally-ingested sesamin in humans and rodents is absorbed and metabolized into various derivatives that circulate in the blood stream [24, 25]. Thus, to understand the molecular mechanisms underlying the physiological effects of sesamin *in vivo*, we focused on the molecular actions of sesamin metabolites. We found that SC1, one of sesamin metabolites by CYP450 *in vivo*, has much stronger anti-inflammatory activities than sesamin itself in the mouse macrophage-like J774.1 cells. Our data also showed that the anti-inflammatory effects of SC1 could be attenuated after the phase-II metabolism with the glucuronidation/sulfation of the catechol moieties. These findings indicate the important role of the catechol groups in the anti-inflammatory effects. However, the activity of di-catechol SC2 is weaker than that of the mono-catechol SC1, although the detailed actions of SC2 were not examined in this study. Similarly, the biological activities of quercetin, a

representative food-derived polyphenol, are generally attenuated after conversion to the metabolites [36].

After the oral intake of sesamin in humans, sesamin catechols were detected in the β-glucuronidase/sulfatase-treated plasma, but not in the intact plasma [24], indicating that sesamin catechols are readily metabolized *in vivo* to the glucuronides and sulfates. Furthermore, the maximum concentration ($C_{max}$) of SC1 in deconjugation enzyme-treated human plasma was approximately 18 times higher than that of SC2 [24, 25], suggesting that the glucuronides and/or sulfates of SC1 but not that of SC2 highly contribute to the physiological effects of sesamin *in vivo*. Consistent with the reports that macrophages have a β-glucuronidase activity [26-28], SC1 glucuronides were deconjugated to accumulate mainly as SC1m in the macrophages. The cellular accumulation of SC1 in the cells treated with SC1 glucuronides was also observed in another mouse macrophage-like cell line, RAW264 (data not shown). The anti-inflammatory activities of SC1 glucuronides significantly increased in a pre-incubation time-dependent manner. The addition of the β-glucuronidase inhibitor completely cancelled the cellular accumulation of SC1 and SC1m and anti-inflammatory effects in the cells treated with SC1 glucuronides. In addition, the COMT inhibitor impaired the cellular accumulation of SC1m with increased SC1 and enhanced the anti-inflammatory effects. These results suggest that sesamin catechol glucuronides are deconjugated to SC1 by the β-glucuronidase activity and most of SC1 is rapidly converted to SC1m by COMT

activity, and that SC1 rather than SC1m mainly acts as suppressor of pro-inflammatory signaling in the cells.

LPS, an outer membrane component of Gram-negative bacteria, can activate the innate immune system to induce the expression of inflammatory mediators in macrophages/monocytes [37]. The activation of TLR4 by LPS recruits myeloid differentiation protein-88 (MyD88) or toll-interleukin-1 receptor domain-containing adapter inducing IFN-β (TRIF) [38]. In the MyD88-dependent pathway, MyD88 activates the downstream signaling components including IκB kinase β (IKKβ) and MAPK, resulting in the activation of the transcription factor NF-κB and AP-1 to induce the expression of the pro-inflammatory mediators including iNOS. On the other hand, in the TRIF-dependent signaling pathway, TRIF activates the IKKε and tumor necrosis factor receptor associated factor (TRAF) family member associated NF-κB activator-binding kinase1 which phosphorylates the interferon regulatory factor (IRF3), resulting in the expression of IFN-β [39, 40]. Then, IFN-β stimulates the Janus-activated kinase (JAK) that phosphorylates and activates STAT1 to induce the expression of the downstream genes including iNOS and IP-10 [40-42]. The activation of the TRIF pathway also leads to the delayed activation of NF-κB and MAPK mediated through the association of TRIF with the receptor-interacting protein-1 [43].

We found that, consistent with the inhibitory potency against the LPS-induced NO production, SC1 and SC1 glucuronides suppressed the LPS-induced iNOS expression, whereas the

LPS-activated MAPK pathway and NF-κB pathway were hardly affected by SC1 and SC1 conjugates, and the LPS-increased IFN-β mRNA expression was significantly suppressed. These data suggest that SC1 and SC1 glucuronides inhibit the LPS-induced NO production through attenuation of the IFN-β/iNOS signaling in the TRIF-dependent pathway. As already described, SC1 could be an active form for the inhibitory effects during the treatment with SC1 glucuronides, however, the detailed inhibitory mechanisms of SC1 remain unclear. Further investigations are needed to clarify the upstream targets of SC1 in the LPS-activated TRIF-dependent pathway.

Since the present study aimed to understand the mechanism of action, we employed 10 and 25 μM as the appropriate treatment concentrations of sesamin metabolites to detect their effects clearly in the *in vitro* experiments. It has been reported that the hepatic sesamin concentration in rats at 1 h after 100 mg/kg sesamin administration is 1.1 μM [44]. Although much higher levels of sesamin metabolites than sesamin were detected in rat liver [25], it is unclear whether the concentration of sesamin metabolites can reach *in vivo* at the concentrations used in the present study. Therefore, the further studies on the *in vivo* concentrations of sesamin metabolites under the normal and inflammatory conditions are required.

In this study, cells were treated with sesamin metabolites in FBS-free medium, because it is known that the proteins in FBS such as an albumin bind to the polyphenols to decrease their bioavailability [45]. Therefore, higher level of sesamin metabolites might be required to exhibit the

biological effects under the condition with the serum compared with under the serum-free condition.

The time-to-maximum concentration $T_{max}$ of sesamin metabolites in human plasma is 5 h, and that sesamin metabolites are detected even at 12-24 h after ingestion of sesamin, though the concentrations are drastically decreased from $C_{max}$ [24]. Furthermore, due to the increased β-glucuronidase-like activity at the inflamed sites [26-28], the sesamin metabolites might be deconjugated to exert the anti-inflammatory effects *in vivo* in a shorter time than *in vitro*. Future efforts should be directed to clarify to what degree the deconjugation-mediated mechanism contributes to the anti-inflammatory effects of sesamin *in vivo*.

In conclusion, we demonstrated the anti-inflammatory effects of sesamin metabolites in mouse macrophage-like J774.1 cells. The scheme for the anti-inflammatory actions of sesamin metabolites is shown in Figure 6. Sesamin catechol conjugates are considered to be the major metabolites of sesamin existing in human plasma after the oral ingestion of sesamin [24]. Our data strongly suggested that SC1 glucuronides rather than the sulfates could inhibit the inflammatory responses through macrophage-mediated deconjugation. Using the COMT inhibitor, we also demonstrated that SC1 could be an active form for the inhibitory effects in the cell treated with SC1 glucuronides. These results may explain, at least in part, the molecular mechanisms for the beneficial health effects of the sesame intake. Our study will provide information for utilization of natural phenolic compounds, including sesamin, to prevent inflammatory diseases.

## ABBREVIATIONS USED

**COMT**, catechol-*O*-methyltransferase; **UGT**, UDP-glucuronosyltransferases; **SULT**, sulfotransferases; **SC1-3GA**, SC1-3-*O*-glucuronide; **SC1-4GA**, SC1-4-*O*-glucuronide; **SC1-3S**, SC1-3-*O*-sulfate; **SC1-4S**, SC1-4-*O*-sulfate

## AUTHOR CONTRIBUTIONS

N.A.K. and Y.K. designed the experiments. N.A.K., Y.L. and Y.K. conducted the experiments and analyzed the data. D.T., Y.O., H.S., K.O. and Y.K. assisted with the experiments and contributed to the discussions. N.A.K. and Y.K. interpreted the experiments and wrote the manuscript.

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## CONFLICT OF INTEREST

D.T., Y.O. and S.H. are employees of Suntory Wellness Ltd., which is a manufacturer of foods that contain sesamin.

## SUPPORTING INFORMATION

**Supporting Information Figure S1.** Chromatogram of HPLC-ECD for medium and standards of sesamin metabolites.

**Supporting Information Figure S2.** Chromatogram of HPLC-ECD for Figure 3A. The peaks of chromatogram are identified based on the data in Supporting Information Figure S1.

**Supporting Information Figure S3.** Chromatogram of HPLC-ECD for Figure 5A. The peaks of chromatogram are identified based on the data in Supporting Information Figure S1. Representative data of three separate experiments are shown.

**Supporting Information Figure S4.** Chromatogram of HPLC-ECD for Figure 5C. The peaks of chromatogram are identified based on the data in Supporting Information Figure S1.

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## FIGURE CAPTIONS

**Figure 1. Metabolic pathway of sesamin.** (A) CYP450-catalyzed metabolism of sesamin to the catechol derivatives. (B) Chemical structures of sesamin metabolites used in this study.

**Figure 2. The effects of sesamin-related compounds on the LPS-induced NO production in macrophages.** The J774.1 cells were treated with the combination of each sesamin-related compound (10 or 25 μM) and LPS (1 μg/mL) for 24 h. After incubation, the nitrite concentration in the cell culture supernatant was determined by the Griess assay. The data are expressed as relative values, with LPS only groups being 100%. The values represent the means ± S.D. of three separate experiments.

**Figure 3. The role of the deconjugation of SC1 conjugates on their anti-inflammatory effects in macrophages.** (A) The J774.1 cells were treated with SC1 conjugates (25 μM) for the indicated hours. The cellular accumulation of SC1, SC1m and each SC1 conjugate was determined by HPLC-ECD. (B) The J774.1 cells were pre-treated with SC1 conjugates (25 μM) for the indicated hours, then stimulated by LPS (1 μg/mL) for 24 h. After incubation, the nitrite concentration in the cell culture supernatant was determined by the Griess assay. The data are expressed as relative values, with LPS only groups at each treatment time being 100%. The values represent the means ± S.D.

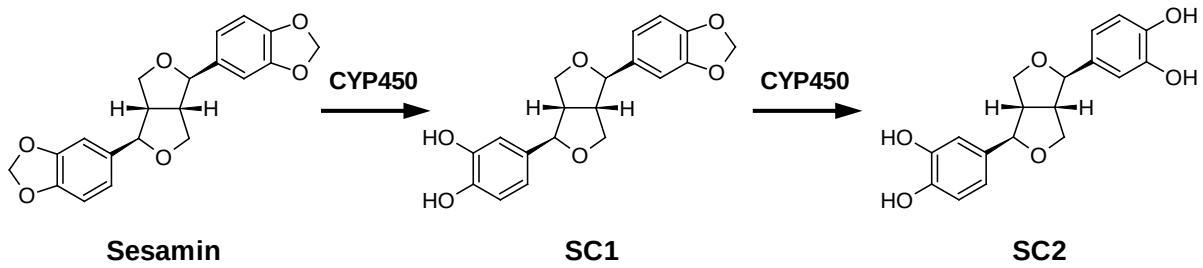
of three separate experiments. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using R software, version 3.4.2. Different letters above the bars indicate significant differences among treatments for each compound ($P$<0.05).

**Figure 4. The effects of SC1, SC1m and SC1 conjugates on the LPS-induced proinflammatory signaling pathway in the macrophages.** (A) The J774.1 cells were treated with the combination of SC1 or SC1m (25 μM) and LPS (1 μg/mL) for 8 h or treated with SC1 conjugates (25 μM) for 24 h prior to the LPS stimulation for 8 h. Whole cell lysates were prepared and a Western blot analysis was performed for iNOS and β-actin. (B) The J774.1 cells were treated with the combination of SC1 (25 μM) and LPS (1 μg/mL) for 30 min. Whole cell lysates were prepared and a Western blot analysis was performed for p-ERK, ERK, p-JNK, JNK, p-p38 MAPK, p38 MAPK, IκB and β-actin. (C) The J774.1 cells were treated with SC1 conjugates (25 μM) for 24 h prior to LPS stimulation for 30 min. Whole cell lysates were prepared and a Western blot analysis was performed for p-ERK, ERK, p-JNK, JNK, p-p38 MAPK, p38 MAPK, IκB and β-actin. (D) The J774.1 cells were treated with the combination of SC1 (25 μM) and LPS (1 μg/mL) for 3 h or treated with SC1 conjugates (25 μM) for 24 h prior to LPS stimulation for 3 h. The relative mRNA level of IFN-β normalized to GAPDH was determined by a real-time RT-PCR. The values represent the means ± S.D. of three separate experiments. Data were analyzed by a one-way ANOVA

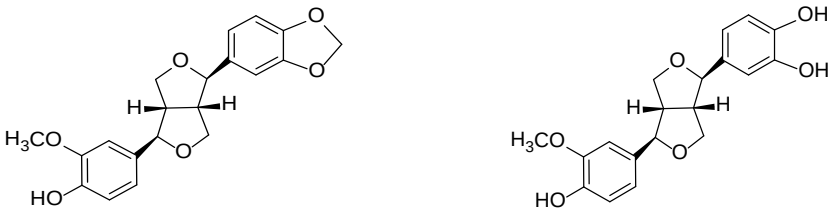
513 followed by Tukey's HSD using R software, version 3.4.2. Different letters above the bars indicate
514 significant differences among treatments for each compound ($P$<0.05).
515
516 **Figure 5. The effects of β-glucuronidase inhibitor and COMT inhibitor on the cellular**
517 **accumulation of SC1 and SC1m and the anti-inflammatory effects induced by the treatment**
518 **of SC1 glucuronides in the macrophages.** (A) Cells were treated with SC1 conjugates (25 μM)
519 in the absence and presence of D-saccharic acid 1,4-lactone (2 mM) for 24 h. D-Saccharic acid 1,4-
520 lactone was added to the medium every 8 h to maintain the activity due to its instability. The cellular
521 accumulation of SC1m and SC1 were determined by HPLC-ECD. (B) The J774.1 cells were pre-
522 treated with SC1 conjugates (25 μM) for 24 h in the absence and presence of D-saccharic acid 1,4-
523 lactone (2 mM). D-Saccharic acid 1,4-lactone was added to the medium every 8 h to maintain the
524 activity due to its instability. The cells were then stimulated by LPS (1 μg/mL) for 24 h and the
525 nitrite concentration in the cell culture supernatant was determined by a Griess assay. (C) The
526 J774.1 cells were pre-treated with SC1 conjugates (25 μM) for 24 h in the absence and presence of
527 3,5-dinitrocatechol (5 μM). The cells were then stimulated by LPS (1 μg/mL) for 24 h and the
528 nitrite concentration in the cell culture supernatant was determined by a Griess assay. The values
529 represent the means ± S.D. of three separate experiments. Data were analyzed by a one-way
530 ANOVA followed by Tukey's HSD using R software, version 3.4.2. Different letters above the bars

indicate significant differences among treatments for each compound ($P$<0.05).

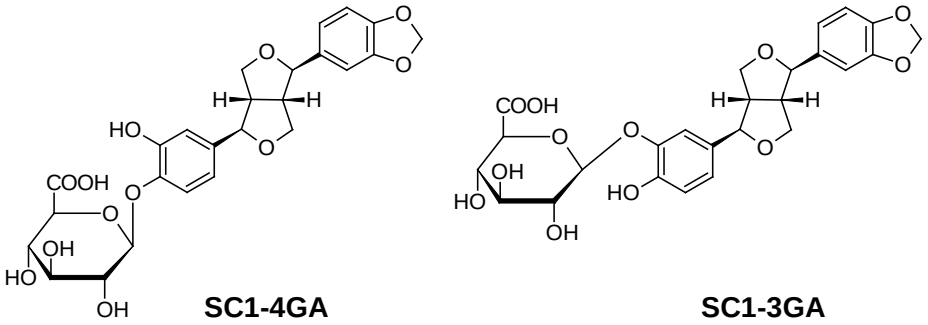
**Figure 6. Proposed mechanism for the anti-inflammation by SC1 conjugates.** Sesamin is converted by the phase-I metabolism into catechol derivatives and further converted by a phase-II metabolism into their glucuronide or sulfate conjugates *in vivo*. SC1 glucuronides are deconjugated to accumulate in the cells mainly as SC1m due to the β-glucuronidase activity and the COMT activity of macrophage. On the other hand, SC1 sulfates are scarcely deconjugated. Pre-treatment of SC1 glucuronides inhibit LPS-induced NO production through attenuation of the IFN-β/iNOS signaling in the TRIF-dependent pathway, but not in the MyD88-dependent pathway. SC1 rather than SC1m could be an active form of the anti-inflammatory effects of SC1 glucuronides.



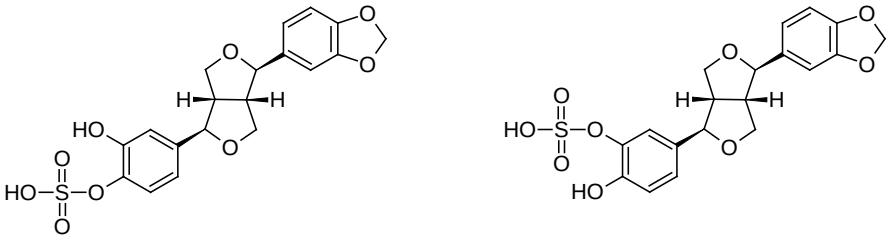

**Sesamin** **SC1** **SC2**



**SC1m** **SC2m**



**SC1-4GA** **SC1-3GA**



**SC1-4S** **SC1-3S**

**Figure 1.**

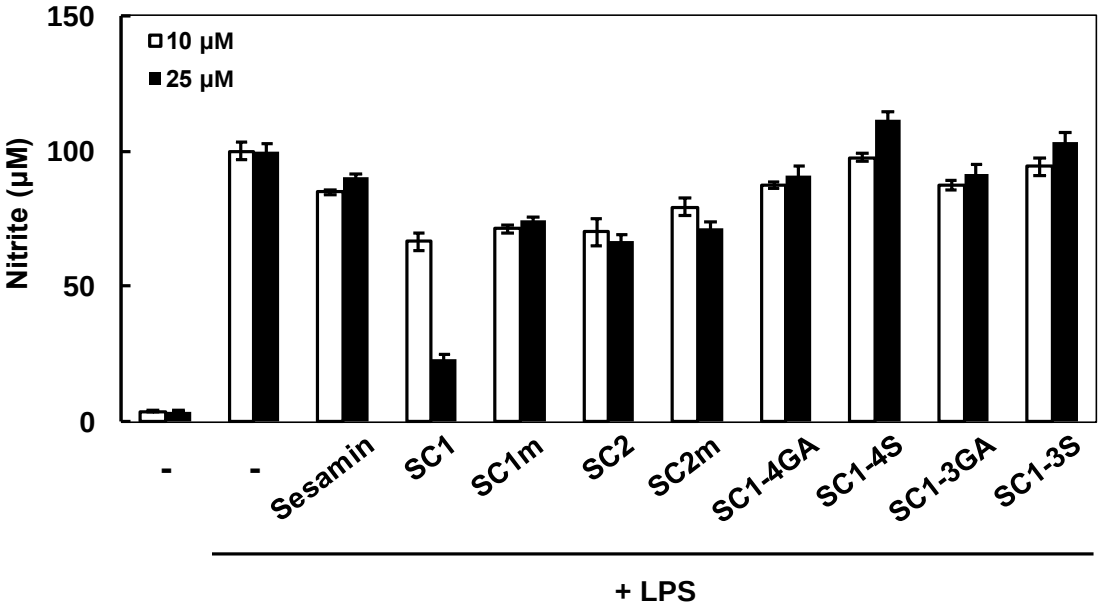

150
10 μM
25 μM
100
Nitrite (μM)
50
0
-
-
Sesamin
SC1
SC1m
SC2
SC2m
SC1-4GA
SC1-4S
SC1-3GA
SC1-3S
+ LPS

**Figure 2.**

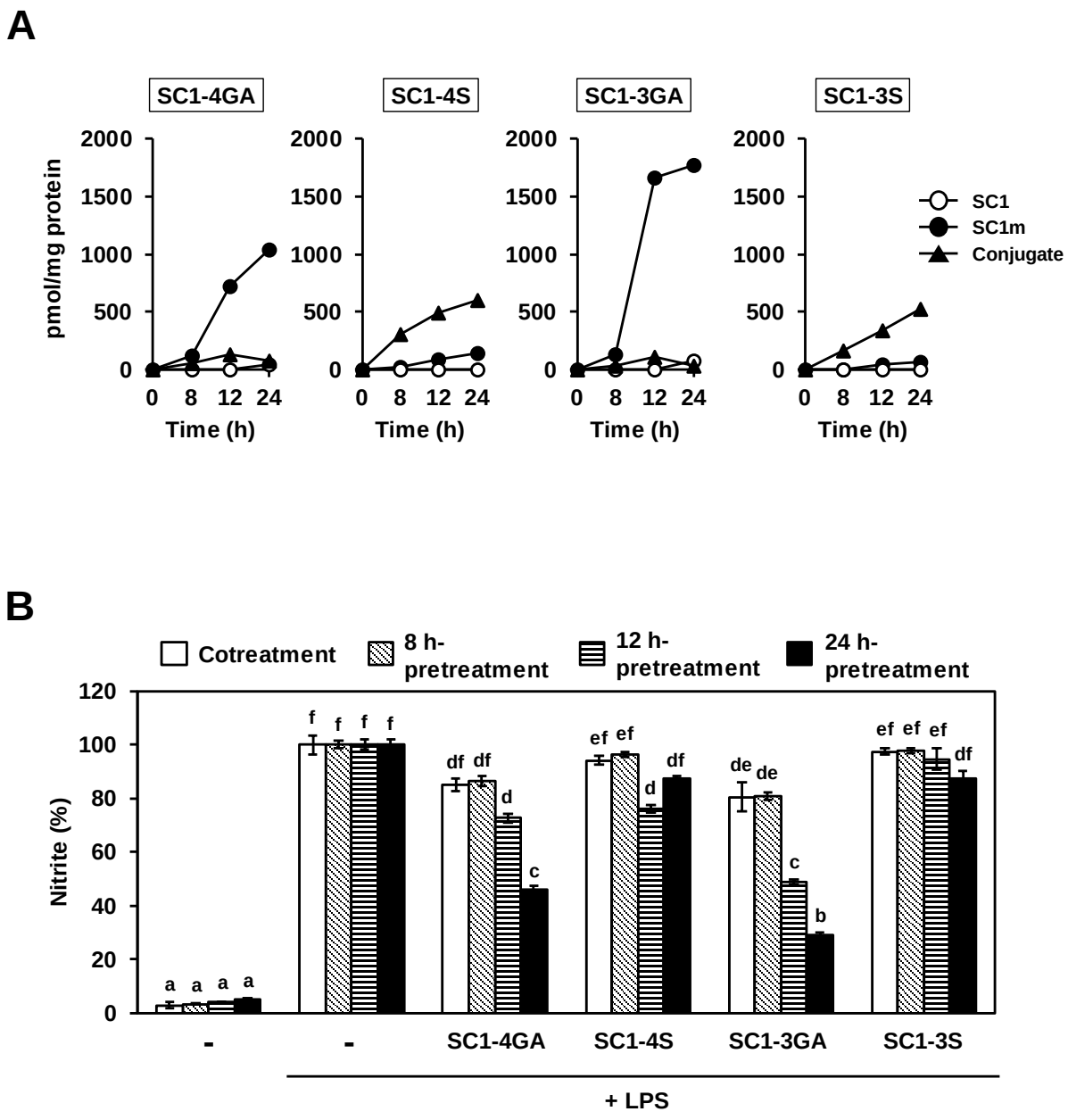

A
SC1-4GA
SC1-4S
SC1-3GA
SC1-3S
pmol/mg protein
2000
1500
1000
500
0
0 8 12 24
Time (h)
SC1
SC1m
Conjugate
B
Cotreatment
8 h-pretreatment
12 h-pretreatment
24 h-pretreatment
Nitrite (%)
120
100
80
60
40
20
0
a a a a
f f f f
df df d c
ef ef d df
de de c b
ef ef ef df
-
-
SC1-4GA
SC1-4S
SC1-3GA
SC1-3S
+ LPS

**Figure 3.**

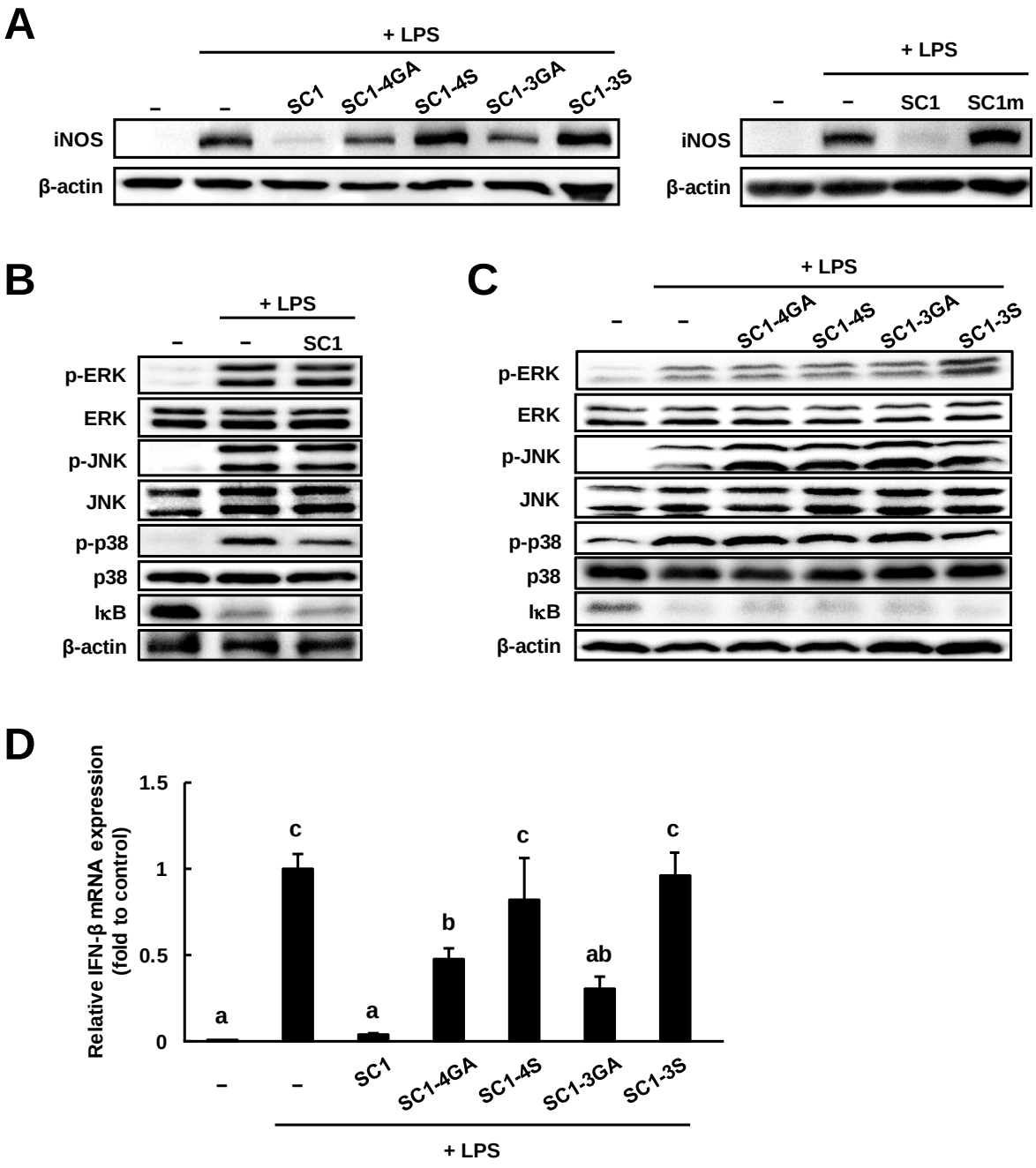

A
+ LPS
– – SC1 SC1-4GA SC1-4S SC1-3GA SC1-3S
iNOS
β-actin
+ LPS
– – SC1 SC1m
iNOS
β-actin
B
+ LPS
– – SC1
p-ERK
ERK
p-JNK
JNK
p-p38
p38
IκB
β-actin
C
+ LPS
– – SC1-4GA SC1-4S SC1-3GA SC1-3S
p-ERK
ERK
p-JNK
JNK
p-p38
p38
IκB
β-actin
D
Relative IFN-β mRNA expression (fold to control)
1.5
1
0.5
0
a c a b c ab c
– – SC1 SC1-4GA SC1-4S SC1-3GA SC1-3S
+ LPS

**Figure 4.**

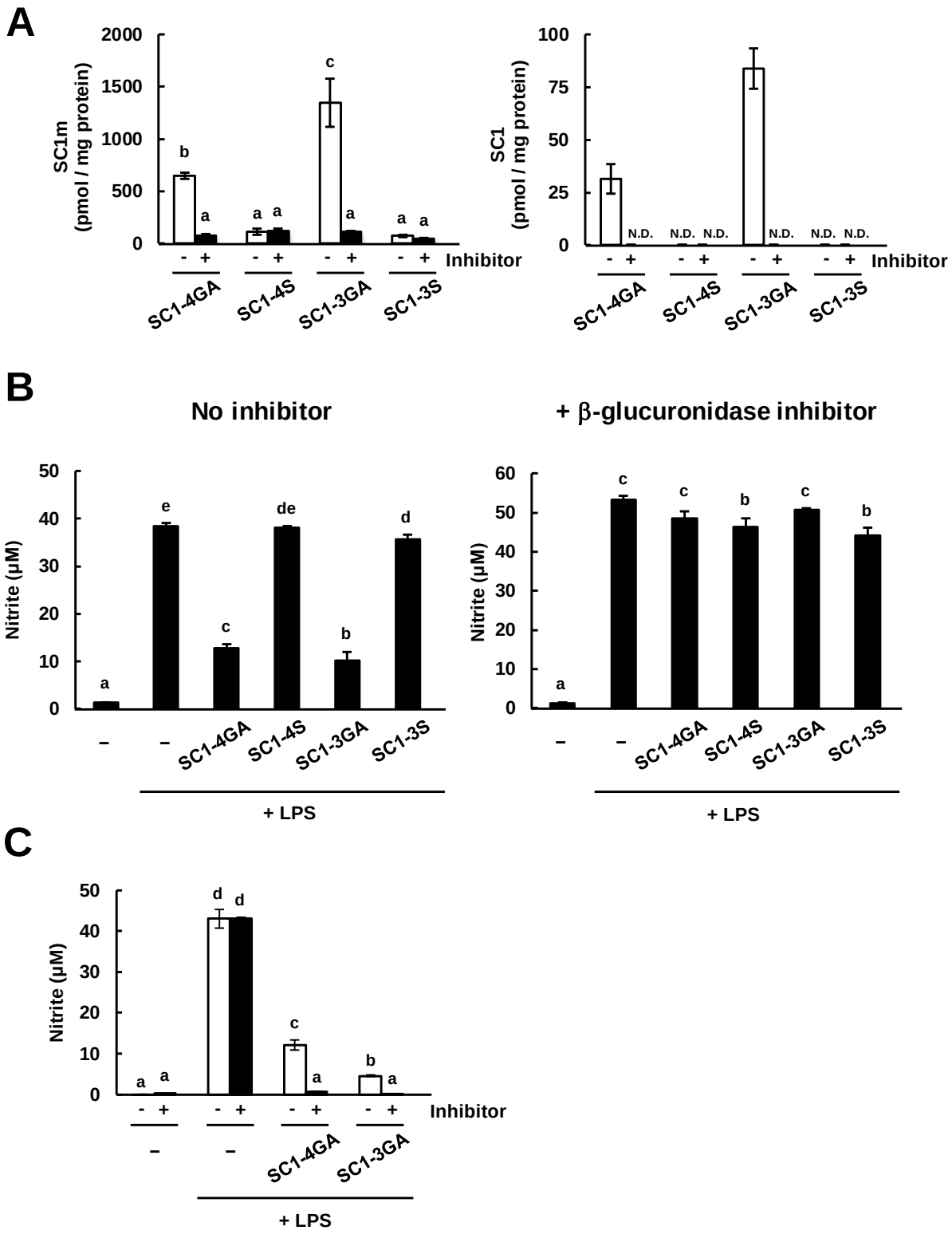

A
SC1m (pmol / mg protein)
2000
1500
1000
500
0
b
a
a a
c
a
a a
- + - + - + - +
Inhibitor
SC1-4GA
SC1-4S
SC1-3GA
SC1-3S
SC1 (pmol / mg protein)
100
75
50
25
0
N.D. N.D. N.D. N.D. N.D. N.D.
B
No inhibitor
+ β-glucuronidase inhibitor
Nitrite (μM)
50
40
30
20
10
0
a
e
c
de
b
d
60
c c b c b
– – SC1-4GA SC1-4S SC1-3GA SC1-3S
+ LPS
C
d d
c
b
a a a a
Inhibitor
– – SC1-4GA SC1-3GA
+ LPS

**Figure 5.**

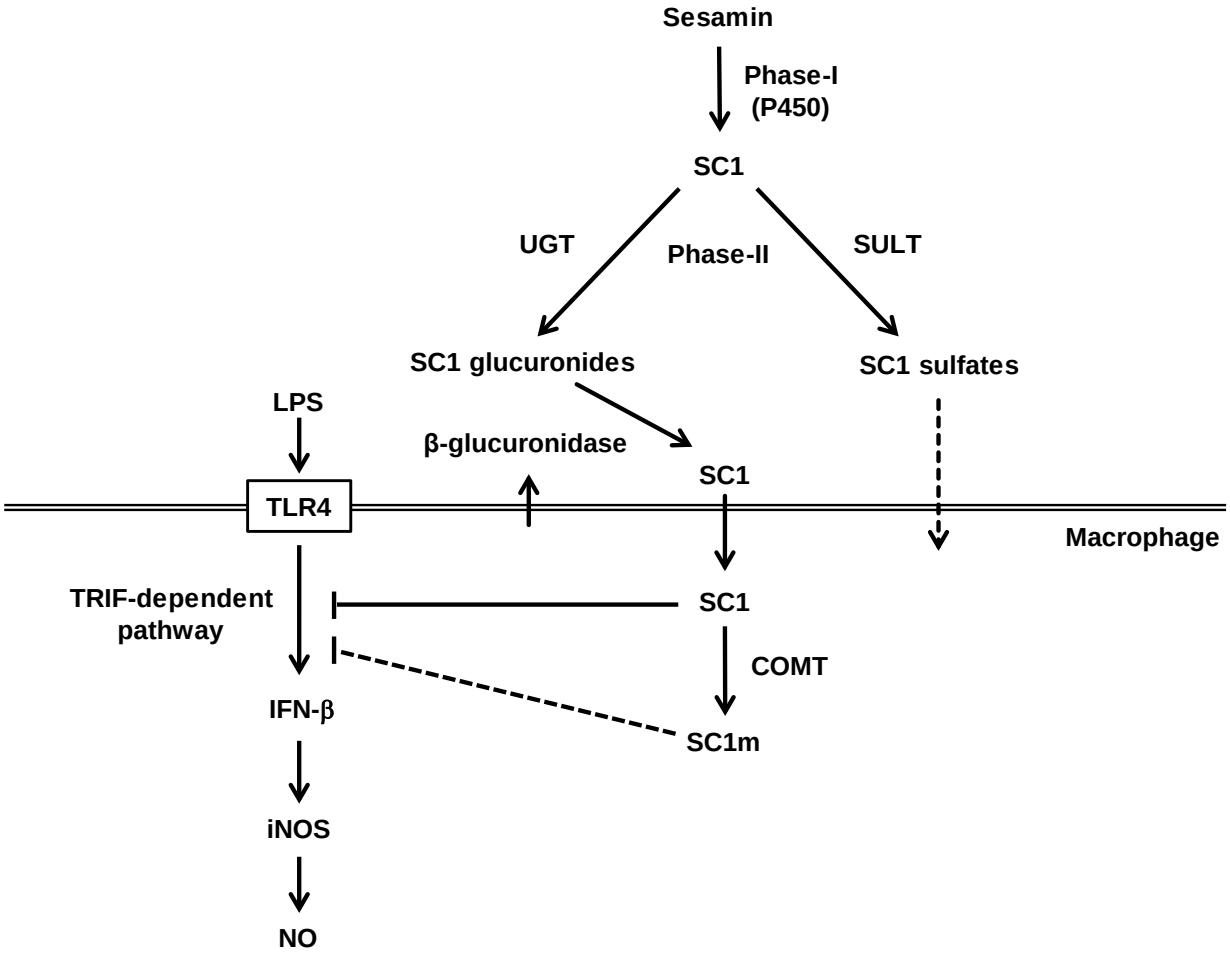

Sesamin
Phase-I
(P450)
SC1
UGT
Phase-II
SULT
SC1 glucuronides
SC1 sulfates
LPS
β-glucuronidase
SC1
TLR4
Macrophage
TRIF-dependent
pathway
SC1
COMT
IFN-β
SC1m
iNOS
NO

**Figure 6.**

## Graphic for table of contents