Short communication

Anti-inflammatory effects of soyasapogenol I-αα via downregulation of the MAPK signaling pathway in LPS-induced RAW 264.7 macrophages

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ABSTRACT

The crude extract of soyasaponins was reported to possess anti-inflammatory activity. We determined the new purity group I saponin, I-αα and I-γα that was isolated from wild soybean (Glycine soja) in terms of its efficacy in protecting RAW 264.7 macrophages from lipopolysaccharide (LPS)-stimuli. Cells were treated with soyasaponin I-αα/I-γα (30–300 μM) and LPS (0.1 μg/mL) for 24 h. Soyasaponin I-αα inhibited nitric oxide (NO) production at 100 μg/mL, while soyasaponin I-γα demonstrated this effect at a higher concentration (200 μg/mL). The expression levels of iNOS and COX-2 enzymes were downregulated by both soyasaponins. Soyasaponin I-αα exerted its effect via the TNF-α and IL-1β cytokines. However, soyasaponin I-γα only inhibited the expression of TNF-α. The inflammatory effect of group I soyasaponin was mainly mediated via the phosphorylation of the p38 and JNK proteins. Collectively, these results suggested the potential anti-inflammatory effects of soyasaponins.

1. Introduction

Inflammation is a physiological response of living tissues against invading microorganisms by a complex network of innate immunity, cellular reactions and chemical signals that is accompanied by the increase of vascular permeability and the activation of leukocytes (Coussens and Werb, 2002). Lipopolysaccharides (LPS), a principal component of the outer membrane of gram-negative bacteria, activate monocytes and macrophages in order to produce pro-inflammatory mediators including nitric oxide (NO) and cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and IL-6. LPS induce mitogen-activated protein kinase (MAPKs) including extracellular signal-regulated kinases (ERK)-1 and −2, c-Jun N-terminal kinase (JNK), and p38 and this interaction can increase inflammatory disease states (Caradonna et al., 2000; Guzik et al., 2003). Therefore, the chemical inhibition of the release of inflammatory mediators from activated macrophages is an important target for the treatment of various inflammatory disorders.

Soyasaponins are major bioactive secondary plant metabolites that are present in soybean (Glycine max) and wild soybean (G. soja). They are generally divided into four main groups, namely A, B, E, and DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrones) and three minor groups, namely H, I, and J depending on the chemical structure of the aglycone (soyasapogenol) (Itabashi et al., 2016). The chemical structures of group I soyasaponins are similar to that of melilotigenin that is a compound identified in the aerial part of Melilotus officinalis (Kang and Woo, 1988) and contains a sugar chain at the C-3 position and a COOH group at the C-29 positions. A total of Six isomers of group I soyasaponins, namely I-αg, I-βg, I-γg, I-αa, I-βa, and I-γa were isolated from soybeans and wild soybean by Krishnamurthy et al. (2014). Soyasaponins possess membranolytic activity in human cells in order to exert several pharmacological effects including in vitro and in vivo antioxidant potential and anti-fungal, anti-bacterial, anti-inflammatory, and anti-cancer activities (Francis et al., 2002; Güçlü-Üstündag and Mazza, 2007; Kang et al., 2005; Sung et al., 1995). Vila-Donat et al. (2015) reported that soyasaponin I exhibited synergistic cytoprotective

Abbreviations: COX-2, cyclooxygenase 2; DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4-pyrones; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinases; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; MAPKs, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PDA, photodiode-array detector

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effects against AOH-induced cytotoxicity in Caco-2 cells. The anti-inflammatory properties of soybean have also been investigated in detail by the inhibition of the transcription of inflammatory cytokine genes via the NF-κB signaling pathway (Kang et al., 2005; Zha et al., 2011). However, no report exists in the literature regarding the anti-inflammatory activity of group I soyasaponin and the exact mechanism by which soyasaponin I-α and I-γα mediate their reported anti-inflammatory effects.

In the present study, the anti-inflammatory activity of soyasaponin I-α and I-γα that were isolated from wild soybean, were investigated by several in vitro assays using LPS-stimulated RAW 264.7 macrophages as a model. The inhibitory effect of soyasaponin I-α on the inflammatory biomarkers such as NO, the expression levels of iNOS, COX-2, and proinflammatory cytokines as well as the phosphorylation levels of MAPK proteins (p38, ERK1/2, JNK) were investigated. The current study therefore not only provides additional evidence on the anti-inflammatory activity of soybean saponins, but also provides a plausible molecular mechanism of anti-inflammatory function.

2. Materials and methods

2.1. Chemicals and reagents

Murine macrophage cell line RAW 264.7 were purchased from the American Type Culture Collection (ATCC TIB71). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (10,000 unit/10,000 μg/mL) was obtained from Invitrogen/ Gibco (Grans Island, NY, USA). Protease inhibitor and phosphatase inhibitor were obtained from Roche (Indianapolis, IN, USA). Trizol reagent and SuperScript III Platinum One-Step qRT-PCR Kit were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Other chemicals and reagents were obtained from Sigma (Missouri, USA). Antibodies for iNOS, COX-2, and β-actin primary antibodies were purchased from Santa Cruz (CA, USA).

2.2. Isolation of soyasaponin

Soybean saponins I-αα and I-γα were purified by the method described in Itabashi et al. (2016) with some modifications. The seed hypocotyls (45 g) were milled and extracted in fivefold volume (v/v) of 80% (v/v) aqueous methanol for 1 h at room temperature. The extracts were concentrated on a rotary evaporator under vacuum at 36°C. The dried extracts (45 mg) were suspended in 645 mL of 1 M KOH for 10 min and subsequently mixed with 64.5 mL of concentrated HCl. The precipitate was collected by centrifugation and resuspended in 645 mL of 0.1 M HCl. After 10 min, the supernatant was removed by centrifugation at 7200 × g for 5 min. This process was repeated three times. For crude saponin solution preparation, the resulting precipitate was resuspended in 645 mL of 40% methanol containing 0.1% acetic acid. The pure group I soyasaponin was collected though reverse-phase C18 open column (2.2 × 30 cm; YMC gel ODS-A, 12 μm, S-75 μm, YMC Co, Ltd., Kyoto, Japan) with elution process from 50% to 80% methanol (250 mL each, containing 0.1% acetic acid). The soyasaponin I-αα extract was dried under freeze drying to powder and kept at −20°C.

2.3. LC-PDA-MS/MS analysis

The purity of group I soyasaponin was analyzed by LC-PDA-MS/MS. A total of 5 μL of sample was applied though an analytical reverse-phase column (Develosil C30-UG-5, 4.6 mm × 150 mm, Nomura Chemical Co., Aichi, Japan) coupled with a photodiode-array detector (PDA) and a tandem mass (LTQ Orbitrap XL; Thermo Fisher Scientific, Waltham MA, USA) at 40°C. The mobile phase consisted of 0.5% formic acid in acetonitrile (A) and 0.5% formic acid in water (B) and the separation was conducted at a flow rate of 150 μL/min. A linear gradient was initiated with 20% solvent A and it was increased to 65% during 45 min. The column was washed by increasing the concentration of solvent A to 100% (v/v) for 5 min followed by 20% solvent A for 15 min. The absorbance was measured at 205 and 292 nm. The soyasaponin contents were calculated from a standard curve on the basis of the peak area of purified saponin I-αα that was measured at 205 nm.

2.4. Cell culture, cell viability assay and NO production

RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO2 at 37°C and the medium was renewed every 2–3 days. Prior to treatment, the RAW 264.7 cells were cultured in 96-well plates for 24 h. The cells were treated with soyasaponin I-αα and/or I-γα (30–300 μM) and LPS (0.1 μg/mL) for 24 h. The cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, USA) assay. Subsequently, the NO levels were measured in the conditioned culture media using the Griess reagent as described by Srisko and Cha (2005).

2.5. Total RNA extraction and reverse transcription-polymerase chain reaction (RT- PCR)

Total RNA was isolated with Trizol reagent according to the manufacturer’s instruction. Total RNA (1 μg) was reverse-transcribed to produce cDNA using a SuperScript III Platinum One-Step qRT-PCR Kit according to the manufacturer’s instructions. The target cDNA was amplified using the following primers: inducible nitric oxide synthase (iNOS), forward 5′-GCACAGCAGAATAATTTTCAGCAG-3′ and reverse 5′-AGCCGACATTCCGGATGAC-3′; cyclooxygenase-2 (COX-2), forward 5′-TTGATGAACTACGTGCAACC-3′ and reverse 5′-TTCATAATGTGAACTGTCGCCAGC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-GGTTGTCCCTCTGACTGAC-3′ and reverse 5′-GTTCGCTGACCAATACTGTGT-3′; TNF-α, forward 5′-GGCTGCCCAGACTCAGT-3′ and reverse 5′-ACTTTCTCCTGTGATGATAGCAAAT-3′; IL-1β, forward 5′-GTCACAAGAACCATTGGCAT-3′; IL-6, forward 5′-GGAGGCTTAAATCCACTGTT-3′ and reverse 5′-TGAATATACAGATGATGAT-3′.

2.6. Western blot analysis

RAW 264.7 cells were washed twice with PBS buffer at 4°C. The cell proteins were extracted in RIPA lysis buffer containing protease and phosphatase inhibitors. The supernatants were collected after centrifugation at 14,000 rpm at 4°C. Equal amounts of protein were separated by 10% SDS-PAGE and were transferred onto a PVDF membrane. 5% skim milk in PBS buffer was used to block nonspecific binding. Subsequently, the membranes were incubated further with primary antibodies of iNOS, COX-2, β-actin, phospho-JNK (p-JNK), p38, phospho-p38 (p-p38) TNF-α, IL-1β and IL-6, for 1 h at room temperature. The blots on the PVDF membrane were visualized with a PowerOpti-ECI (Animal Genetics Inc, Gainesville, FL, USA) detection system according to the recommended procedure.

2.7. Statistical analysis

All data were expressed as mean ± SD of at least three independent experiments. The statistical significance was performed using SPSS software (SPSS Inc., Chicago, IL) via analysis of variance (ANOVA), followed by Tukey’s multiple comparison tests. Differences at P < 0.05 were considered to be significant.
3. Results

3.1. Inhibitory effects of group I soyasaponins on cell survival and NO production

The chemical structures of soyasaponin I-α and I-γ are shown in Fig. 1. RAW 264.7 macrophage cells were treated with group I soyasaponins at a concentration range of 30–300 μg/mL with or without LPS (0.1 μg/mL) for 24 h. Both group I soyasaponins at a concentration of 300 μg/mL were not significantly cytotoxic against RAW 264.7 macrophages as shown in Fig. 2A and B. As shown in Fig. 2C and D, NO production following LPS-treatment increased significantly (P < 0.001) compared with that noted in uninduced cells. Soyasaponin I-α significantly inhibited LPS-induced NO production by 71.2 ± 2.32%, 38.89 ± 4.23% and 24.23 ± 2.53% at 100, 200 and 300 μg/mL compared with the LPS-induced control group. RAW 264.7 macrophages treated with I-γ exhibited NO inhibition by 30.54 ± 4.75% and 26.39 ± 2.11% at 200 and 300 μg/mL, respectively. The inhibitory activity of I-α on NO synthesis was higher than that of I-γ, although the concentration required for that activity was half to that required in the case of I-γ. Concurrently, dexamethasone at 0.1 μM, which is a specific inhibitor of iNOS activity, decreased NO production by 73% (Fig. 2C and D).

3.2. Inhibitory effects of group I soyasaponins on iNOS and COX-2 expression

LPS could significantly increase the expression of iNOS and COX-2 proteins in LPS-induced RAW 264.7 cells compared with uninduced cells (Fig. 3A and B). Soyasaponin I-α (300 μg/mL) decreased...
significantly LPS-stimulated iNOS and COX-2 expression levels up to 62.1 ± 4.20% and 71.53 ± 4.65%, respectively compared with the LPS-treated control group. As shown in Fig. 3B, I-γ significantly inhibited LPS-induced NO production in a concentration-dependent manner with an IC_{50} value of 242.3 ± 21.4 μg/mL. β-actin, one of the ‘housekeeping’ proteins used as an internal control, was not affected significantly by compound treatment. Moreover, iNOS and COX-2 mRNA expression levels were determined using qRT-PCR analysis. The mRNA levels of iNOS and COX-2 in RAW 264.7 cells were markedly up-regulated following stimulation with LPS. Group I soyasaponins significantly down-regulated the expression levels of iNOS and COX-2 mRNA in a concentration-dependent manner (Fig. 3A and B). However, group I soyasaponins alone (50 μM) did not affect iNOS and COX-2 expression.

3.3. Inhibitory effects of group I soyasaponins on inflammatory cytokines

The ability of group I soyasaponins to induce the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) via iNOS was examined in LPS-induced RAW 264.7 macrophage cells. RT-PCR analysis indicated that the mRNA levels of these cytokines increased following LPS treatment. The presence of soyasaponin I-α or I-γ attenuated the upregulation of TNF-α and IL-1β in a dose-dependent manner (Fig. 4 and Fig. 5). Soyasaponin I-α and soyasaponin I-γ exerted 72.65 ± 0.102% and 78.23 ± 0.024% inhibition at concentrations of 300 μg/mL compared with the LPS-treated control group (Fig. 4B and 5B). Soyasaponin I-α significantly suppressed the production of IL-1β production at 300 μg/mL, while soyasaponin I-γ was ineffective with regard to the stimulation of IL-1β expression (Fig. 4C and 5C). In contrast to IL-1β, both group I soyasaponins did not affect inhibition of IL-6 production compared with LPS-induced cells. These results indicate that group I soyasaponins acted by preventing expression of these cytokines involved in the inflammatory process at the transcriptional level.

3.4. Inhibitory effects of group I soyasaponins on MAPK/ATF-2 signaling pathway

The phosphorylation of ERK1/2, JNK and p38 proteins in RAW 264.7 macrophage cells was induced significantly in cells treated with LPS (Fig. 6). Soyasaponin I-α significantly inhibited the phosphorylation of p38 and JNK proteins in a dose-dependent manner compared with LPS-induced cells, whereas ERK was not affected following treatment of the compound. Similarly, a concentration-dependent decrease in p38 and JNK production was further observed following soyasaponin I-γ treatment, with the exception of I-γa inhibiting JNK production by 67.23 ± 0.421% at 300 μg/mL.

4. Discussion

The anti-inflammatory activity of a number of active components isolated from juices, crop, or plant food products has been previously reported (Gasparrini et al., 2017; Kim et al., 2012, 2013). Soybean flour and cake were previously investigated by several research groups (Dia et al., 2009; Kang et al., 2008; Kao et al., 2007). The crude extract of soybean saponins was reported to significantly inhibit the PGE2, NO, TNF-α and MCP-1 production via the down regulation of the expression of COX-2 and/or iNOS to the mRNA and protein levels (Kang et al., 2005; Zha et al., 2011). However, the diverse biological effects of soyasaponins depend on their chemical structure (Rao and Gurfinke, 2000; Shi et al., 2004). In order to validate the health benefits of two new group I soyasaponins isolated from wild soybean (*Glycine soja*) (Fig. 1), we focused on the molecular mechanism underlying the anti-inflammatory activity of group I soyasaponin in LPS-treated murine macrophage RAW 264.7, which is widely accepted model for inflammation studies.

NO levels were used as an index of -stimulated inflammatory response in macrophages due to their involvement in various inflammatory diseases (Wong et al., 1996). We found that both group I soyasaponins exhibited potent inhibitory effect on the production of NO. However, the chemical structure of I-α only differs from I-γa by the presence of an additional D-glucose unit attached to the C-3 (OH) position (Fig. 1). The difference between the cytotoxic effects of these compounds may probably be attributed to the additional D-glucose moiety. This compound exhibited non-significant cytotoxicity at 300 μg/mL indicating that the decrease in NO levels was not attributed to its cytotoxic effect. The lowest-observed-adverse-effect level of soyasaponin was estimated to 707.2 mg/kg b.w./day for male and 751.8 for female rats, respectively (Cho et al., 2009).

Macrophage cells express solely iNOS and COX-2 proteins for responding to neurotoxic and/or inflammatory stimuli, which can further lead to an increase in NO production in neuronal diseases (Surh et al., 2001; Vane and Botting, 1995). In order to explain the inhibitory effects of group I soyasaponins on NO production, the effects of these...
compounds on LPS-induced iNOS levels were investigated. In the present study, the expression of both proteins correlated with their mRNA levels. Soyasaponin I-αa treatment significantly decreased the levels of iNOS enzyme at its highest concentration (300 μg/mL) while soyasaponin I-γa exerted this effect at 100 μg/mL compared with the only LPS-treated control group. The addition, of both of group I soyasaponins suppressed the COX-2 production at 100 μg/mL, suggesting that the inhibitory effects of group I soyasaponins on NO release might be due to the decrease in the expression levels of iNOS and COX-2 mRNA. These effects subsequently reduced the protein levels of these two markers and ultimately suppressed NO production in LPS-activated RAW 264.7 macrophages. The crude soy saponin was also reported to possess anti-inflammatory activity by down-regulating COX-2 and/or iNOS (Kang et al., 2005).

The increased production of inflammatory cytokines such as TNF-α, IL-6 and IL-1β indicates inflammatory neuronal damage (Carlson et al., 1999). In the present study, soyasaponin I-αa and I-γa significantly inhibited LPS-induced production of TNF-α, as mentioned in a previous study (Kang et al., 2005; Zha et al., 2011). Moreover, soyasaponin I-αa was found to be effective on stimulating the production of the IL-1β in RAW 264.7 cells (Jeon et al., 2000; Jung et al., 2003). Similar results with regard to the inhibition of IL-1β were obtained with other bioactive compounds (Jeon et al., 2000; Jung et al., 2003). The cytokine IL-1β is an important mediator of fever, hypotension, and the acute phase reaction (Dinarello, 1996). These results indicate that the anti-inflammatory activity of soybean might depend on their saponin compositions.

The phosphorylation of MAPK proteins is a major signaling event for the majority of cellular processes. The proteins ERK, p38 and JNK play a role in the inflammatory processes in LPS-induced macrophages (Kaminska, 2005; Obata et al., 2000). The activation of these proteins might regulate the inflammatory gene expression that includes TNF-α and IL-1β. These cytokines are required for the expression of iNOS and COX-2 following LPS stimulation (Obata et al., 2000; Surh et al., 2001). Further investigations are necessary in order to fully address the inhibitory effects of group I soyasaponins on iNOS and cytokines, and their effects on the phosphorylation of MAPK proteins. In the present study, group I soyasaponins markedly reduced the phosphorylation of p38 MAP kinase and JNK but not ERK. Similarly, iNOS, COX-2, TNF-α and IL-1β inhibition by group I soyasaponins was evident in LPS-stimulated RAW 264.7 macrophages possibly due to the inhibition of p38 and JNK phosphorylation under inflammatory conditions.

![Fig. 4. Effect of soyasaponins I-αa on the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in RAW 264.7 macrophages that were induced with LPS for 24 h. The values are expressed as mean ± SD (n = 3). ###P < 0.001 compared with uninduced cells, *P < 0.05 and **P < 0.01, significantly different from LPS-treated RAW 264.7 macrophage cells.](image)

![Fig. 5. Effect of soyasaponins I-γa on the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in RAW 264.7 macrophages induced with LPS for 24 h. The values are expressed as mean ± SD (n = 3). ###P < 0.001 compared with uninhibited cells, *P < 0.05, significantly different from LPS-treated RAW 264.7 macrophage cells.](image)
In conclusion, group I soyasaponins exerted anti-inflammatory activity on NO production in LPS-stimulated macrophages via the downregulation of both protein and mRNA levels of iNOS and COX-2 and via the inhibition of the phosphorylation of p38 and JNK proteins. Although the inhibitory effect of BFNM on NO production is comparable to the drug dexamethasone, these data collectively provide new insight into the neuroprotective actions of soyasaponins. This evidence may explain the anti-inflammatory activity of soybean, possibly due to composition of saponin compounds.

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