Identification of the Constituents for Nrf2 Activation and NF-κB Suppression in Dangguisoo-san

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Previously, we showed that Dangguisoo-san (DGSS), an herbal formula that has been traditionally used for the treatment of blood stagnation, is also applicable for inflammatory lung diseases. Activation of Nrf2, an anti-inflammatory transcription factor, and suppression of NF-κB, a pro-inflammatory transcription factor, were suggested as an underlying mechanism. However, the constituents responsible for these activities remain unidentified. To this end, we prepared the water extracts of the 9 constituents of DGSS and tested for their effect on Nrf2 by using an Nrf2-Luciferase reporter cell line and western blot analysis. Results show that *Carthamus tinctorius L.*(CT), one of the 9 constituents of DGSS, strongly activated Nrf2. Similarly, when measured the effect of the 9 constituents on NF-κB by using an NF-κB-Luciferase reporter cell line and western blotting for nuclear p65, indicative of activated NF-κB, most constituents were capable of suppressing NF-κB in various degrees. However, CT and *Cyperus rotundus L.* (CR) strongly suppressed NF-κB activity elicited by LPS. Of note, CT activated Nrf2 and suppressed NF-κB strongly as well. Our results contributes to corroborating the anti-inflammatory effects of DGSS by identifying CT and CR as two major herbs responsible for activating Nrf2 and suppressing NF-κB. These results suggest that CT and CR represent some of the effects of DGSS in the regulation of inflammation.

Key words : anti-inflammation, Nrf2, NF-κB, c*arthamus tinctorius, cyperus rotundus* lipopolysaccharide

Introduction

Inflammation is a complex response of immunity and plays an essential role in fending off cellular damage. However, uncontrolled, excessive inflammatory reactions can cause various inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, asthma, pulmonary fibrosis, and cancer[1]. Therefore, regulation of inflammation can be a therapeutic target, which warrants an intensive research.

Macrophages are a key effector cell in regulation of inflammation. For instance, macrophages sense immunogens and initiate inflammation by using various Toll-like receptors (TLRs) on the cell surface. They recognize lipopolysaccharide (LPS), a cell wall component of Gram negative bacteria, through TLR4 and release various pro-inflammatory mediators. Engagement of LPS to TLR4 triggers a series of signaling cascades, resulting in activation of NF-κB. NF-κB is a major pro-inflammatory transcription factor that regulates the expression of various molecules involved in inflammation, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), growth factors, bioactive lipids, reactive oxygen intermediates, and the short-lived free radical[2,3]. Recently, it has been reported that the inflammatory response generates oxidative environment, activating nuclear factor-E2-related factor 2 (Nrf2), a member of the cap'n'collar family of basic leucine zipper transcription factors[4]. Activated Nrf2 accumulates in the nucleus where to bind to the antioxidant responsive element (ARE), resulting in the expression of phase 2 detoxification and antioxidant enzymes such as glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H:quinine oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1)[5]. Accumulating evidence also shows that Nrf2 plays essential roles in regulation of inflammation, although the precise underlying mechanisms remain unclear[6]. Nevertheless, these studies suggest that differential regulation of Nrf2 and NF-κB activities can be excellent therapeutic targets for the treatment of various inflammatory diseases.

In Korean traditional medicine, medicinal herbal extracts

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have long been prescribed and often effectively treated various inflammatory diseases[7]. Despite potentially significant health benefits, the lack of understanding the underlying mechanisms for the effect has hampered a wide usage of them in clinic. Thus, uncovering the underlying mechanisms is considered as an imperative for a broad application of Korean traditional medicine. Dangguisoo-san (DGSS) is an herbal formula composed of 9 plants, Carthamus tinctorius L.(CT), Cinnamomum cassia Presl (CC), Caesalpinia sappan L. (CS), Paeonia lactiflora Pall (PL), Linder astrychnifolia Fernandez-Villar (LA), Angelica gigas Nakai (AG), Cyperus rotundus L. (CR), Prunus persica Batsch (PB), Glycyrrhiza uralensis (GU), and has been prescribed for the treatment of blood stagnation[8]. In our previous study, we provided evidence that DGSS is applicable for lung inflammation, the effect of which is associated with differential regulation of Nrf2 and NF-κB[9]. In this study, to obtain an insight on the role of each constituent of DGSS in suppressing inflammation, we determined the effects of each constituent on Nrf2 activation and NF-κB suppression.

Materials and Methods

1. Water Extraction of Herbal Plants

Herbal plants composed of DGSS, Carthamus tinctorius L.(CT), Cinnamomum cassia Presl (CC), Caesalpinia sappan L. (CS), Paeonia lactiflora Pall (PL), Linder astrychnifolia Fernandez-Villar (LA), Angelica gigas Nakai (AG), Cyperus rotundus L. (CR), Prunus persica Batsch (PB), Glycyrrhiza uralensis (GU), were purchased from Kwang myung dang Natural Pharmaceutical Co. Ulsan, Korea. The medicinal herbs were authenticated by Dr. Su-In Cho, who is professor of Division of Pharmacology, School of Korean Medicine, Pusan National University. Sixty gram of each herbal plants was boiled in 1 L of distilled water in an Herb Extractor (Dae-Woong Co, Korea) for 2 h, yielding final 200 ml of herbal plants. After centrifugation, the supernatant was harvested in a sterile condition and lyophilized through evaporation under reduced pressure at -80 ℃, yielding 4.6 to 5.5 g of a final product. DGSS was composited as shown in Table 1.

2. Reagents and Antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TLR4-specific Escherichia coli LPS was purchased from Alexis Biochemical (San Diego, CA, USA). All antibodies used in this study were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3. Cell Culture

RAW 264.7 cells was obtained from ATCC (American Type Culture Collection, Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (200 mg/L) (Hyclone; Logan, UT, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 100 U/ml penicillin and 100 ㎍/ml streptomycin. The cells were cultured and maintained in a humidified incubator at 37 ℃ and 5 % CO_2 prior to experiment.

4. MTT assay

The cytotoxicity caused by herbal plants was assessed with MTT-based colorimetric assay. In brief, after Griess reaction, MTT solution (2.0 mg/ml) was added to each well of cells cultured in a 96 well plate. At 4 h after incubation at 37 ℃ in a CO_2 cell culture incubator, the supernatant were removed, and formazan crystals formed inviable cells were measured at 540nm with a microplate reader. The percentage of living cells was calculated against untreated cells.

5. Western Blot Analysis

Total cell extracts of 5×10^6 cells were prepared as described previously[10]. Nuclear proteins were isolated by NE-PER nuclear extraction kit and the manufacture's protocol (Thermo Scientific, IL, USA). The amounts of proteins were measured by Bradford (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins were fractionated by SDS-PAGE and then transferred to PVDF membrane (Bio-Rad Laboratories). Blots were blocked for at least 1 h with 5% non-fat dry milk prior to incubation with polyclonal antibodies for Nrf2, and lamin A/C at 4℃ overnight. After incubation with secondary antibodies conjugated with HRP for 1 h at room temperature, the bands of interest were revealed by chemiluminescence (SuperSignal® West Femto, Thermo Scientific).

6. Reporter Constructs, Reporter Cell Line, and Luciferase Assay

Measuring Nrf2 transcriptional activity was performed with an Nrf2 reporter cell line that contains a proximal 1kb-long promoter of a murine NQO-1 gene, as described previously[11]. From genomic DNA isolated by QIAmp DNA Mini Kit (Qiagen) and the instructions of the manufacture, the proximal 1kb-long promoter of a murine NQO-1 gene, where an Nrf2 binding site locates, was amplified by PCR with a pair of primers: 5′ - GCTATGTGGACCAGTCTGG - 3′ and 5′ - GGCTCCAGATGTTGAGGGA - 3′. The PCR product was

verified by sequencing and subsequently cloned into pGL4.17 [luc2/Neo] vector (Promega). The resultant vector, NQO-1[luc/Neo], was stably transfected with lipofectamin LTX (Invitrogen) into RAW 264.7 cells, and candidate Nrf2 reporter cell lines were selected under G418 (Invitrogen). The Nrf2 reporter cell line was tested for its responsiveness to Sulforaphane, a well-documented Nrf2 activator. To measure NF-κB transcriptional activity, we created RAW 264.7 cells that were stably transfected with an NF-κB reporter construct. The reporter construct harbors four tandem copies of a 36-base enhancer from the 5′ HIV-long terminal repeat (containing two NF-κB binding site, GGGACTTTCC) placed upstream of the HSV minimal thymidine kinase promoter, which were cloned into pEGFPLuc (BD Biosciences Clontech). The resultant vector, NF-κB[luc/Neo], was stably transfected with lipofectamin LTX (Invitrogen) into RAW264.7 cells, and selected under G418 (Invitrogen). Luciferase activity was measured by a luciferase assay kit (Promega) and the instructions of the manufacturer, and normalized by the amount of total proteins of the cell extract.

7. Statistical Analysis

Data is presented as the mean ± SEM (Std. Err.) of at least three separate experiments. For comparison among groups, paired or unpaired T tests and one-way analysis of variance (ANOVA) tests were used (with the assistance of InStat, Graphpad Software, Inc., San Diego, CA, U.S.A.). P values less than 0.05 was considered statistically significant. All experiment was performed at least three times independently.

Table 1. Constituents of DGSS

Scientific name	Herbal name	Amount (g)
Angelica gigas Nakai	Angelicae gigantis Radix	5.625
Paeonia lactiflora Pall	Paeoniae Radix	3.75
Lindera strychnifolia Fernandez-Villar	Linderae Radix	3.75
Caesalpinia sappan L.	Sappan Lignum	3.75
Cyperus rotundus L.	Cyperi Rhizoma	3.75
Carthamus tinctorius L.	Carthami Flos	3.0
Prunus persica Batsch	Persicae Semen	2.655
Cinnamomum cassia Presl	Cinnamomi Cortex	2.25
Glycyrrhiza uralensis Fisch	Glycyrrhizae Radix et Rhizoma	1.875
Total		30.405

Results

1. DGSS activates Nrf2 but suppresses NF-κB.

Since DGSS was reported to ameliorate acute lung inflammation by activation of Nrf2 and suppression of NF-κB[9], we would like to confirm this. First, we examined whether DGSS activates Nrf2, a master transcription factor that is known to prevent from acute lung inflammation. RAW 264.7 cells were treated with various amounts of DGSS. At 16 h after treatments, the nuclear fraction of the treated cells was fractionated, and Nrf2 activation was analyzed by western blotting of nuclear Nrf2. As shown in Fig. 1A, nuclear Nrf2, indicative of activated Nrf2, was detectable with 10 μg/ml of DGSS, which became apparent in the treatments of 50 μg/ml of DGSS, suggesting that DGSS indeed activates Nrf2. Similarly, we measured the effect of DGSS on NF-κB activity. We used an NF-κB-luciferase reporter cell line derived from RAW 264.7. Treatment of the cell line with TLR4 specific LPS (100 ng/ml) for 16 h yielded a strong luciferase activity, indicating that LPS treatment activates NF-κB mediated transcription in the reporter cell line (compare 1st and 3rd columns). However, pre-treatment of the cell line with DGSS for 16h significantly suppressed the NF-κB activity induced by LPS treatment (4th column). These results show that DGSS activated Nrf2 but suppressed NF-κB activities, corroborating our published results.

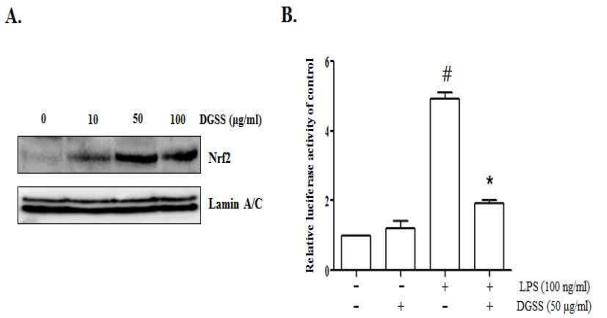

Fig. 1. Effect of DGSS on Nrf2. (A) RAW 264.7 cells, was treated with various amounts of DGSS (10, 50, and 100 μg/ml) for 16h. Nuclear fractions of the treated cells were analyzed by western blotting for Nrf2. For ensuring equal loading, the membrane was stripped and reprobed for lamin A/C. (B) An NF-kB reporter cell line was treated as in (A), and cytoplasmic proteins were harvested for luciferase activity. The activity was normalized by the amount of the cytoplasmic proteins. The experiment was repeated at least three times independently, and data represent the mean ± SEM of three experiments. #P was less than 0.05, compared to untreated control; *P was less than 0.05, compared to LPS treated.

2. Identification of a herbal plant that activates Nrf2

Because of DGSS differential effects on Nrf2 and NF-κB, we attempted to identify an herbal plant accounting for the effects. Since 9 different herbal plants comprise DGSS, we prepared the individual extract of 9 constituents of DGSS and tested whether each herbal extract activate activates Nrf2. First, we determined a cellular toxicity by performing MTT assay. Since we measured the effect of DGSS in the amount of 50 μg/ml, RAW 264.7 cells were treated with 50 μg/ml of *Carthamus tinctorius L.*(CT), *Cinnamomum cassia Presl* (CC), *Caesalpinia sappan L.* (CS), *Paeonia lactiflora Pall* (PL), *Linder*

astrychnifolia Fernandez-Villar (LA), *Angelica gigas Nakai* (AG), *Cyperus rotundus L.* (CR), *Prunus persica Batsch* (PB), or *Glycyrrhiza uralensis* (GU) for 16 h prior to MTT assay. As shown in Fig. 2, herbal plants did not affect the viability of RAW 264.7 except CC. When tested with 25 μg/ml, the cytotoxicity by CC was not detected (data not shown). Thus, in the study, the effects of each constituent of DGSS on Nrf2 and NF-κB were tested with 25 μg/ml in final concentration.

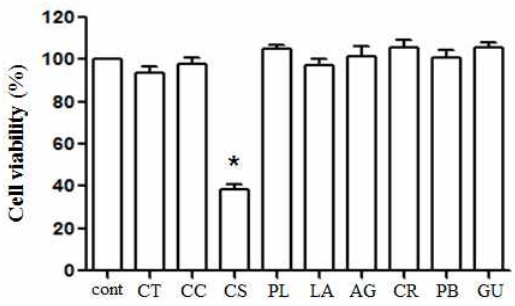

Fig. 2. Effect of herbal plant extracts on the cell viability. Cytotoxicity of herbal plant extracts on RAW 264.7 cells was measured by MTT assay. The cells (106 cells) grown to 80% confluence were treated with 50 μg/ml each extract for 16 h. No toxicity was observed except CS that showed significant cellular toxicity. Data represent the mean±SEM of three independent measurements. *P was less than 0.05, compared to untreated control.

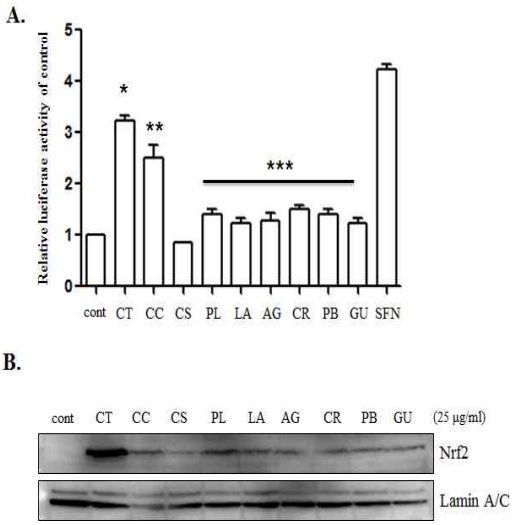

Fig. 3. Effect of Herbal plant extracts on Nrf2 activation. (A) A luciferase assay was performed with the Nrf2 reporter cell line treated with 25 μg/ml of constituents of DGSS, along with SFN (5 μM) for 16h. The activity was normalized by the amount of cytoplasmic proteins. Data represent the mean ± SEM of three independent experiment. *,**,**P were less than 0.05 compared to untreated control. (B) The nuclear Nrf2 was analyzed by western blotting. For ensuring equal loading, the membrane was stripped and reprobed for lamin A/C.

Next, to determine Nrf2 activation induced by each herbal plant extract, we used Nrf2 responsive reporter cell line derived from RAW 264.7 cells. The cells were treated with 25 μg/ml of CT, CC, CS, PL, LA, AG, CR, PB, or AG for 16h. The cytoplasmic fraction was prepared for measuring luciferase activity. As shown in Fig. 3A, most of the constituents of DGSS induced various degrees of luciferase activities. suggesting Nrf2-dependent transcription except CS (4th column). Among the extracts that activated Nrf2, CT and CC showed strong luciferase activity, compared to others (2nd and 3rd columns). Since the reporter construct in the cell line contains various transcriptional binding sites, it is highly likely that the luciferase activity is also influenced by other pathways activated by an herbal extract. Thus, we further confirm the Nrf2 activation by western blot analysis. RAW 264.7 cells were treated with each of 9 constituents of DGSS, as described above, and the nuclear fraction of the treated cells were isolated and analyzed by western blotting for the nuclear Nrf2. As shown in Fig. 3B, similar to Fig. 3A, most constituents induced nuclear localization of Nrf2, although the degree of activation varies. Interestingly, CT was the most strong inducer of Nrf2. Taken together, these results suggest that CT is the major constituent in activating Nrf2.

3. Identification of a herbal plant that suppresses NF-κB

Since DGSS also suppressed NF-κB activity, we determined which herbal plants account for it. For the study, we used the NF-κB luciferase report cell line. Similar to Fig. 3, the reporter cells were treated with 25 μg/ml of CT, CC, CS, PL, LA, AG, CR, PB, or AG, and subsequently further treated with LPS (100 ng/ml). At 16h after LPS treatment, the cytoplasmic fractions of the treated cells were isolated for luciferase assay. As shown in Fig. 4A, LPS induced NF-kB activity (2nd column), showing the responsiveness of the reporter cell line to LPS. However, unlike DGSS, nine constituents of DGSS affected NF-κB activity in various degrees. CT, CC, CS LA, and CR suppressed NF-κB significantly (3rd, 4th, 5th, 7th, and 9th columns). PB and GU also suppressed it, albeit not statistically significant (10th 11th columns). On the other hand, PL and AG did not suppress the activity (6th and 8th columns).

To further confirm these results, we performed western blot analysis for p65, a subunit of NF-κB, translocated into the nucleus. Since the nuclear translocalization of the protein occurs within 15 min after LPS treatment, we treated RAW 264.7 cells with LPS (100 ng/ml) for 15 min and extracted the nuclear fractions of the treated cells for western blotting of p65. The results showd that, similar to Fig. 4A, CT, CC, CS, LA, and CR suppressed the nuclear localization of p65, suggesting that most constituents in DGSS have a capability of suppressing NF-kB. However, CT treatment showed the least nuclear p65 (3rd lane) follow by CR- treated, suggesting that CT and CR are strong suppressors of NF-κB activity in DGSS.

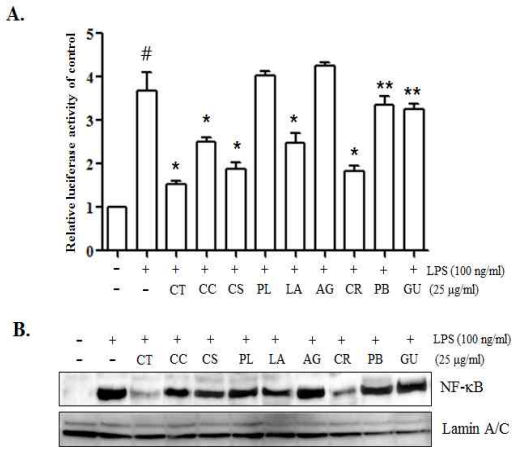

Fig. 4. Effect of Herbal plant extracts on NF-κB. (A) The NF-κB reporter cells were treated with 25 μg/ml of each extract prior to LPS treatment for 16h. Cytoplasmic fractions of the treated cells were used for luciferase activity. Data represent the mean ± SEM of three independent experiments. *P was less than 0.05. **P was above 0.05. #P was less than 0.05, compared to untreated control. (B) The nuclear fractions of the NF-κB reporter cells treated as in (A) were analyzed by western blotting for NF-κB. For ensuring equal loading, the membrane was stripped and reprobed for lamin A/C.

Discussion

Inflammation is a protective innate immune response[12], but regulation of inflammatory reactions is essential because aberrant inflammatory reactions are closely related to various inflammatory diseases including cancer, autoimmune diseases, diabetes, and neuronal diseases[13]. Therefore, regulation of inflammation could be an important measure for the effective treatment of inflammation-associated diseases. In many Asian countries, the extracts of herbs, such as Aralia continentalis[7], Cutellaria baicalensis[14], and Phellodendri chinensis[15], have been using to treat those diseases as part of traditional medicine. Despite the extensive history of the safe and effective usage of medicinal herbs, the lack of understanding the mechanisms by which the herbal treatments execute their effects has hampered the integration of the traditional remedies into mainstream medicine.

Recently, a relationship between blood-stasis and inflammation has been proposed[16]. Experimental evidence suggests that endotoxemia is treatable by "Clearing away Heat-Toxin drugs" that are prescribed in Chinese medicine[17]. In line with these results, our previous study showed that the anti-inflammatory effect of DGSS, a typical herbal formula for removing blood stasis, is associated with activation of Nrf2, an anti-inflammatory transcription factor, and suppression of NF-κB, a pro-inflammatory transcription factor[9]. Since it is not clear which constituents of DGSS are responsible for these effects, in this study, we sought to identify a key constituent in DGSS accounting for the activation of Nrf2 and the suppression of NF-κB. Each extract of nine constituents comprising DGSS were prepared and tested for its effects on Nrf2 and NF-κB. Our results show that CT was the most strong activator of Nrf2 and that while most constituents were capable of suppressing NF-κB activity, CT and CR most strongly suppressed NF-κB activity. Our results are concordant with previous studies showing that CT activates Nrf2 and suppress NF-κB[18] and that CR suppresses NF-κB[19]. However, unlike the study published by other group, where (+)-Nootkatone and (+)-valencene from rhizomes of CR increased the expression of heme oxygenase-1 (HO-1) presumably via Nrf2[20], our results show that CR marginally affected Nrf2 activation. While we do not understand the reason behind this inconsistency, it is likely due to the differences in experimental system between two studies. In addition, since we used a water extract of CR, where the concentrations of (+)-Nootkatone and (+)-valencene are likely low, it is possible that high amounts of CR could activate Nrf2 and thus increase the expression of HO-1.

Nrf2 is a key transcription factor in protecting from various inflammatory diseases, as evidenced by seminal reports showing that genetic deletion of Nrf2 in mice exacerbate acute lung inflammation, smoke-induced emphysema and asthma[21-24]. Given our results showing that CT activated Nrf2 and that CT and CR suppressed the transcriptional activity of NF-κB, a key transcription factor that promotes inflammation[25], it seems that CT and CR were the major constituents of DGSS in suppressing inflammation, likely representing the effect of DGSS on inflammation. It is of note that CT alone had dual activities, Nrf2 activation and NF-κB suppression, performing a strong anti-inflammatory function. Therefore, it would be interesting to study whether the prescription composed of only CT, or CT and CR can replace DGSS in the treatment of blood stagnation and its associated diseases, for which DGSS has been prescribed. Nevertheless, our results suggest that anti-inflammatory activity stems mostly from the two constituents of DGSS.

Although CT and CR seem to be the key constituents of DGSS, it is unclear how they affect Nrf2 and NF-κB. It is apparent that each constituent of DGSS contains a plethora of chemical compounds. In fact, molecular profiles of most constituents of DGSS have been published, although the biological and pharmacological functions conferred by each chemical compound await extensive and thorough studies. Since regulation of Nrf2 activation is primarily carried out by Keap1, an inhibitor that binds to and mediates a continual degradation of Nrf2[26], it is conceivable that some of the chemical compounds in CT or CC formed adducts of Keap1, activating Nrf2. Alternatively, the chemical compounds activate serine kinases, including PKC, MAPK and PERK, which

phosphorylate and activate Nrf2[27-29]. Considering that CT is a potent activator of Nrf2 and a strong suppressor of NF-kB, it is possible that CT contain a chemical compound that can be developed as a potent anti-inflammatory drug. It is, of course, possible that CT contains several compounds affecting Nrf2 and NF-κB independently. Nevertheless, to this end, the identification of the compounds in an herb accounting for these activities is required.

Conclusion

In this study, we sought to identify a constituent of DGSS responsible for activation of Nrf2 and suppression of NF-κB. We found that while CT activated Nrf2, CT and CR suppressed NF-κB. In addition, we show that CT had a dual role in activation of Nrf2 and suppression of NF-κB. Our results contribute to corroborating the effect of DGSS on the activation of Nrf2 and the suppression of NF-κB. Our results suggest that CT, CS, and CR are basic constituents representing the anti-inflammatory effect of DGSS.

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