Diallyl Disulfide (DADS), a Constituent of Garlic, Inactivates NF-κB and Prevents Colitis-Induced Colorectal Cancer by Inhibiting GSK-3β

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Abstract

There is a strong belief that garlic has medicinal properties and may even reduce the risk of developing certain cancers including those of the gastrointestinal tract. The chemopreventive effects of garlic may be attributed to the anti-inflammatory properties of the sulfur-containing constituents of garlic, which includes diallyl disulfide (DADS). Here, we demonstrate that DADS prevented cololectal tumorigenesis in a mouse model of colitis-induced colorectal cancer. Supplementation with 85 ppm of DADS (60 mg daily human equivalent dose) in the diet of FVB/N mice treated with chemical carcinogen azoxymethane (AOM) and colonic irritant dextran sodium sulfate (DSS) resulted in the reduction in tumor incidence, tumor number, and tumor burden by 21.54%, 47.3%, and 66.4%, respectively. Further analysis revealed that mice fed the DADS-supplemented diet resolved the initial DSS-induced inflammation faster than those on the control diet, preventing prolonged inflammation and cellular transformation. Subsequent mechanistic studies in vitro suggest that DADS chemopreventive effects are mediated through NF-κB signaling. When SW480 colorectal cancer cells were treated with DADS, NF-κB nuclear localization and activity were diminished. Interestingly, NF-κB suppression was found to be dependent on DADS inhibition of GSK-3β, a positive regulator of NF-κB. Inhibition of GSK-3β and loss of nuclear NF-κB activity were also observed in vivo in AOM/DSS-treated mice fed a diet supplemented with 85 ppm DADS. Our results indicate that DADS can prevent tumorigenesis by suppressing inflammation, a process largely involving GSK-3β inhibition and consequential reduction in NF-κB nuclear localization. Cancer Prev Res; 9(7); 607–15. ©2016 AACR.

Introduction

Colorectal cancer is the third leading cause of cancer-related mortality in the United States for both male and female (1). Inflammation has been known to play a pivotal role in the initiation and progression of various tumors including those in the colon (2–4). Individuals with intestinal inflammatory conditions such as Crohn disease and ulcerative colitis are at increased risk for developing colorectal cancer (4–7).

There are many bioactive food components with anti-inflammatory activity that are being investigated in relation to the management and treatment of inflammatory bowel conditions that may provide a feasible and safe way to reduce colorectal cancer risk (8, 9). The sulfur-containing constituents derived from garlic; including diallyl disulfide (DADS) are such examples that have been previously reported to exert anti-inflammatory effects (10). DADS has been demonstrated to alleviate inflammation in preclinical models of inflammatory bowel disease by inhibiting the production of proinflammatory cytokines, including IL6 (11).

The NF-κB is a proinflammatory transcription factor that is hypothesized to promote tumorigenesis by regulating the expression of genes that are involved in cell proliferation, apoptosis, and metastasis (12–14). NF-κB is constitutively active in patients with inflammatory bowel disease and may, in part, explain the increased incidence of colorectal cancer observed in these individuals (15). While NF-κB has become a promising target for anticancer therapy, the complexity of NF-κB regulation has made it challenging to develop agents to suppress its activation. The canonical pathway involves various steps including the degradation of the inhibitor of NF-κB (IκB) followed by nuclear translocation of NF-κB and activation of gene transcription (16). A variety of agents have been shown to indirectly suppress NF-κB by influencing key regulatory elements of the canonical pathway, including kinase subunits of the IKK complex, which is responsible for the degradation of IκBs (17).

Glycogen synthase kinase-3 (GSK-3) is a serine threonine kinase comprising two homologous proteins, GSK-3α and GSK-3β, which are products of highly similar but different genes (18). GSK-3β is better characterized than GSK-3α and possesses both tumor suppressor and tumor-promoting activity depending on the cellular context (19, 20). Classically, GSK-3β is regarded primarily as a tumor suppressor due to its role in the Wnt signaling cascade where it phosphorylates β-catenin resulting in its ubiquitin-mediated degradation, thus preventing its
nuclear translocation and subsequent transcription of proto-oncogenes (18). However, emerging evidence has shown that GSK-3β can also promote cancer by activating the NFκB signaling cascade by enhancing the transcriptional activity of NFκB in the nucleus (20). Studies have shown that inhibiting GSK-3β attenuates proliferation and induces apoptosis in colorectal cancer cells (19, 21).

Epidemiologic evidence has been suggestive of the benefit of garlic intake in preventing colorectal cancer with at least one interventional study revealing modest benefits of garlic in preventing colorectal adenoma reoccurrence (22). To further investigate the anti-inflammatory activity of garlic and its ability to prevent colorectal cancer, we assessed the effect of one of the sulfur-containing constituents DADS in a mouse model of colitis-induced colorectal cancer. We found that dietary supplementation with DADS significantly reduced colorectal tumorigenesis in mice treated with azoxymethane (AOM) and dextran sulfate sodium (DSS). DADS exerted anti-inflammatory effects resolving DSS-induced inflammation in these mice. DADS also inhibited NFκB activation and nuclear translocation, which we were able to demonstrate, was dependent on the inhibition of GSK-3β in human colorectal cancer cells.

Materials and Methods

Animal studies

All mouse experiments were conducted according to methods described previously (23) and agreed to and regulated by the Animal Care and Use Committee of the National Cancer Institute (Frederick, MD). Briefly, 6-week-old pathogen-free FVB/N mice were injected with AOM (Sigma) intraperitoneally at a dose of 10 mg/kg body weight in 0.1 mL of saline (day 0, Fig. 1A). Ten days later, mice were treated with 2% DSS (36,000 to 50,000 kDa, MP Biomedicals LL), dissolved in normal drinking water (reverse osmosis–purified water) for 7 days and then switch back to normal drinking water (day 17, Fig. 1A). Three days after completion of DSS treatment (day 20, Fig. 1A), the mice were evenly sorted by change in body weight into four diet groups to ensure effects of the DSS were evenly represented in each of the different diets. Mice were single caged and either maintained on AIN-93G–purified diet from Harlan Teklad (day 20) or started on an AIN-93G diet supplemented with 21, 42, or 85 ppm DADS (Sigma; Supplemental Table S1). Mice were allowed to eat ad libitum. Food consumption and body weight were measured throughout the entire study. Mice were euthanized on day 52 (Fig. 1A) and colon tissue was harvested and stained with 0.2% methylene blue in PBS. The tumors visualized by staining were counted using dissecting microscope (Fig. 1B).

DADS human equivalent dose and tolerance

The daily human equivalent dose (HED) was calculated using body surface area normalization method in which the animal dose is converted to a HED by the following formula (24).

\[
\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times \left(\frac{\text{Mouse Km factor}}{\text{Human Km factor}}\right)
\]

where Animal dose = Diet concentration (mg/kg) × animal mass (kg)/food consumption (kg/week(mean))/7.

The Km factor for mouse and human is 3 and 37, respectively. The HED of 21, 42, 85 ppm dietary DADS was determined to be 15, 30, and 60 mg/kg daily, respectively, and was well tolerated with no obvious toxicity.

Histopathology

Colonic tissue was processed utilizing methods described previously (23). For histologic analysis, colorectal sections were stained with hematoxylin and eosin (H&E) and blindly evaluated by a board-certified pathologist and diagnosed applying the criteria of a consensus report on murine colon tumors (25). Briefly, tumors were characterized as an adenoma or carcinoma and semiquantitatively scored on a number of criteria including; severity of infiltrate (1, mild; 2, moderate; 3, severe), distribution of leukocytes (1, focal/locally; 2, multifocal; 3, diffuse), distribution of epithelial erosion (1, focal/locally extensive; 2, multifocal; 3, diffuse), severity of necrosis (1, mild; 2, moderate; 3, severe), dysplasia (1, low-grade multifocal; 2, low-grade multifocal; 3, high-grade unifocal; 4, high-grade multifocal). A score of 0 was assigned for each criterion not represented in the section. The summation of the five criteria yielded a total disease score per mouse.

IHC

IHC was performed utilizing methods described previously (23). The primary antibodies and dilutions were as follows: pGSK3α/β (Cell Signaling Technology), 1:50; Ki-67, 1:100; pNFκB P65 (Abcam), 1:100; CD45 (BD Biosciences), 1:50. All slides were digitized using Aperio Scanscope CS2 (Leica Biosystems) and evaluated using the Aperio analysis package, which includes highly advanced algorithms for quantifying % of nuclear staining, and staining intensity quantification ranging from 0 (negative) to 3 (strong positive).

Cell culture

Human colorectal cancer SW480 cell line was obtained from ATCC, which prior to distribution was tested and authenticated by the manufacturer using Identifiler STR genotyping. Cells were cultured and stored according to the manufacturer’s instructions and used between passages 5 to 10. Once resuscitated, cell lines were cultured in RPMI supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in humidified air with 5% CO2 never passaged over a period exceeding 2 months to ensure authenticity. DADS (>80% purity by high-performance liquid chromatography to <10%–20% Diallyl sulfide) and LY294002 (Cell Signaling Technology) was used in cell culture experiments.

Western blot analysis

Approximately, 30 to 80 mg of nuclear or cytoplasmic protein that was isolated from SW480 colorectal cancer cells using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) was loaded and separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the following antibodies overnight at indicated dilutions: pNFκB P65 1:500, 1:1,000, NFκB (P65), 1:1,000, pGSK3α/β 1:1,000, pGSK3α/β 1:500, pIKBa/S9, 1:2,000, GSK-3β, 1:1,000, COX-2, 1:1,000, plKBα/β 1:1,000, IKBa, 1:1,000, pIKBa/IKKb 1:250, 1:1,000, IKKe/IKKβ, 1:1,000, pAkt S473 1:1,000, Akt, 1:1,000, β-Catenin, 1:1,000, pβ-Catenin S33/37/41 1:1,000 (Cell Signaling Technology). Each membrane was probed with P84 (Gene Tex) to ensure consistent loading of nuclear protein and β-actin, 1:5,000 (Sigma) to ensure consistent loading of cytoplasmic protein.
Soft agar assay

SW480 cells were seeded onto 100-mm plates and pretreated with either DMSO vehicle (<1%) or DADS (5 μmol/L) for 24 hours. Cells were trypsinized and seeded at 30,000 cells in 2× RPMI media. Cell suspension was added 1:1 with 0.5% agarose (2 mL/well in a 6-well plate) and constituted the top layer. The bottom layer consisted of 2 mL of 1.2% agarose. The cells were maintained in an incubator for 14 days and the colonies were scanned and counted with GelCount (Oxford Optronix Ltd).

Cell proliferation assay

The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay was performed according to methods described previously (26).

Statistical analyses

All data are presented as the mean ± SD. The significance of the difference between groups was evaluated by one-way ANOVA or Student t test, and multiple comparisons with Prism 5.0 software. P < 0.05 was considered to be statistically significant.

Figure 1.
Diallyl disulfide (DADS) inhibited AOM/DSS-induced tumorigenesis. A, timeline showing experimental time points. Male and female FVB/N mice were treated with a single injection AOM (10 mg/kg) on day 0 followed by one cycle of 2% DSS from day 10 to 17. On day 20, mice were grouped by weight loss and randomly started on experimental diets that included AIN93G diet supplemented with 0, 21, 42, or 85 ppm of DADS. Mice were euthanized on day 52. B–D, analysis of colorectal tissue collected at day 52. B, representative macroscopic images of colons stained with 0.2% methylene blue in PBS displaying contours of adenomatous polyps as shown by arrows. Mice supplemented with 85 ppm DADS developed less adenomatous polyps compared with control. C, adenomatous polyps per mouse were counted and presented with SE. Tumor burden in mm³/mouse was estimated by geometric mean and expressed with SE. *; P < 0.05; **; P < 0.01 indicates a significant difference compared with control group as determined by t test. D, histopathology showed a decrease in inflammation in DADS-fed mice. Colorectal tissue collected on day 52 were stained with hematoxylin and eosin and semiquantitatively scored for the degree of inflammation and colonic tissue damage produced after AOM/DSS exposure; severity of infiltrate (1, mild; 2, moderate; 3, severe), distribution of leukocytes (1, focal/local; 2, multifocal; 3, diffuse), distribution of epithelial erosion (1, focal/local/extensional; 2, multifocal; 3, diffuse), severity of necrosis (1, mild; 2, moderate; 3, severe), dysplasia (1, low-grade unifocal; 2, low-grade multifocal; 3, high-grade unifocal; 4, high-grade multifocal). A score of 0 was assigned for each criterion not represented in the section. *, P < 0.05; **; P < 0.01 indicates a significant difference compared with control group as determined by t test. E, diallyl disulfide (DADS) inhibited inflammation in vivo. Cohorts of AOM/DSS mice on respective experimental diets were weighed approximately every 2 to 3 days, and the values are expressed as change of weight (kg) from that of the previous measurement. *, P < 0.05; **; P < 0.01 indicates a significant difference compared with AOM/DSS control group as determined by t test. F, photomicrographs (20x) of colon sections at day 52 stained with hematoxylin and eosin (top row) and anti-CD-45 (1:50) IHC (bottom row) revealed decreased inflammation in mice on diet supplemented with 85 ppm DADS. Scale bar, 100 μm.
Results

DADS prevented tumorigenesis in AOM/DSS–treated mice

In the dinitrobenzenesulfonic acid (DNBS)-induced colitis mouse model, DADS treatment for 2 days was able to exert an anti-inflammatory effect reducing the expression of proinflammatory cytokines (11). To determine whether the anti-inflammatory properties of DADS translate to a decreased risk of developing colorectal cancer, we fed an equal number of FVB/N male and female AOM/DSS-treated mice with a diet supplemented with 21, 42, or 85 ppm DADS (HED of 15, 30, or 60 mg/kg, respectively) daily for 5 weeks. FVB/N mice exposed to the chemical carcinogen AOM and gut irritant DSS present with a number of adenomatous lesions localized in the large bowel with the potential to progress to adenocarcinomas (27, 28). Supplementation starting 3 days post-DSS administration with 42 and 85 ppm DADS resulted in a significant decrease in the total number of adenomatous polyps by 34.3% ($P < 0.01$) and 47.3% ($P < 0.01$), respectively (Fig. 1C). In addition, 21, 42, 85 ppm DADS supplementation resulted in a significant reduction in tumor burden by 24.2% ($P < 0.05$), 33.4% ($P < 0.01$), and 66.4% ($P < 0.01$), respectively (Fig. 1C). Although in this study, tumorigenesis did not progress to adenocarcinomas as seen in previous studies, histopathologic analysis revealed that the lesions in mice treated with 85 ppm were less transformed with the dysplasia frequently less than high-grade ($P < 0.01$; Fig. 1D). Furthermore, 6 of 23 (24%) of mice treated with...
DADS Prevents Colorectal Cancer in Mice

Figure 3.
DADS inhibited GSK-3. Western blot analysis of cytoplasmic (left) and nuclear (right) SW480 CRC cell lysates probed with Abs against p-S21/9-GSK-3β, GSK-3β, total β-catenin, phosphorylated β-catenin at S33/S37/T41, Akt, and p-S473-Akt. Cells were treated with vehicle (DMSO), 2.5 μmol/L, 5 μmol/L, 10 μmol/L, or 20 μmol/L DADS for 24 hours. DADS induced inhibition of GSK-3 as shown by increased phosphorylation at S21/9 which was more pronounced in the nuclear fraction. DADS also reduced expression and phosphorylation at S33/S37/T41 of β-catenin, a major substrate of GSK-3β, confirming the suppressive effect of DADS on GSK-3β activity. β-Actin was used as a cytoplasmic loading control and p84 displayed as nuclear loading control. Each Western blot analysis shown is a representative example of at least three independent experiments.

85 ppm DADS were tumor-free with normal pathology, compared with 1/22 (4.5%) in control (data not shown). The overall response to DADS did not vary between male and female test mice. Taken together, DADS dietary supplementation prevented the development of colitis-induced colorectal cancer in AOM/DSS mouse model.

DADS shortened the recovery time from DSS-induced weight loss and thereby prevented prolonged inflammation

FVB/N mice exposed to 2% DSS will develop colitis, which has been extensively characterized by various techniques including MRI and the examination of the severity of inflammation correlates with loss in body weight (28, 29). In animals fed the control diet, exposure to one cycle of 2% DSS showed a reduction in body weight peaking at 7 to 9 days post-DSS exposure (Fig. 1E) with a gradual recovery as indicated by regain of structural shape and the decrease in CD45+ positive weight gain. Dietary supplementation with DADS exposure (Fig. 1E) with a gradual recovery as indicated by reduction in body weight peaking at 7 to 9 days post-DSS exposure (Fig. 1E) with a gradual recovery as indicated by

DADS inhibited proliferation of SW480 colorectal cancer cells in vitro

To elicit the molecular mechanisms of DADS chemopreventive activity, the human CRC SW480 cell line was treated with varying concentrations of DADS (2.5 μmol/L, 5 μmol/L, 10 μmol/L, 20 μmol/L, 40 μmol/L) for 24 hours. The SW480 CRC cell line was chosen based on the observed responses in a previous study with DADS (31) and because the cell line is well characterized in terms of the biochemical and chromosomal properties (32). In addition to the loss of APC on chromosome 5, activating point mutation of KRAS and C-MYC amplification, SW480 cells harbor mutations in proinflammatory mediators, including, TGFβ (33) and platelet-derived growth factor (PDGF; ref. 34), thereby making the SW480 in vitro studies comparable with the AOM/DSS model of inflammatory bowel disease. DADS treatment resulted in a significant concentration-dependent inhibition of cancer cell viability in SW480 cells (Fig. 2A). In contrast, DADS did not inhibit the viability of nontumor cell line HEK293 (data not shown).

DADS inhibited NF-κB nuclear translocation and reduced PTGS2 (COX-2) expression in vitro

NF-κB activation is essential for development of colonic adenomas in AOM/DSS-treated mice (35). NF-κB contributes to tumorigenesis by inducing genes that are proinflammatory such as PTGS2 (COX-2) (36). The P65/p50 heterodimer is the most abundant NF-κB complex in the cell and is regulated by the so-called canonical pathway, with phosphorylation of P65 at serine 536 being important for its activity (37, 38). In the current study, we found a dose-dependent reduction in phosphorylated-NF-κB (P65)S536 in the nucleus of SW480 cells treated with DADS, which corresponded with a reduction in PTGS2 (COX-2) protein expression in the cytoplasm (Fig. 2B).
Activation of the canonical NF-κB pathway, indicated by phosphorylation of IκBα and IKKβ, appears to be unaffected by DADS treatment (Fig. 2C). The DADS-dependent reduction of phosphorylated P65 in the nucleus and the corresponding increase in phosphorylated P65 in the cytoplasm suggest DADS treatment is blocking nuclear translocation of P65.

DADS inhibited GSK-3 activity

Glycogen synthase kinase-3 (GSK-3) protein that consists of two isoforms (α and β) is present in both the cytoplasm and nucleus and is an important regulator of the inflammatory process. GSK-3(α/β) is activated by phosphorylation of Tyrosine (279/216) and is inactivated by phosphorylation of Serine (21/9). GSK-3β knockout mice are embryonically lethal (21). GSK-3β promotes the activation of NF-κB leading to the survival of cells exposed to TNFα or tumor cells in which the NF-κB pathway is constitutively active (39). DADS treatment inhibited GSK-3β in the nucleus as shown by increased expression of pGSK3βS9 in the nuclear fractions (Fig. 3). Furthermore, GSK-3β was fully phosphorylated at Serine 9 even with the lowest dose of DADS (2.5 μmol/L; Fig. 3) suggesting that the suppressive effects of DADS on GSK-3β activity could be direct and/or highly sensitive. The inactivation of GSK-3β can occur by a number of kinases most notably by Akt (40). There was a modest increase in Akt activity (pAktS473) in the cytoplasm at the highest dosages of DADS; however, GSK-3β inhibition is seen even at the lowest dose of DADS and therefore it is unlikely that GSK-3β inhibition is mediated through Akt (Fig. 3). Thus, it is possible that another kinase is responsible for GSK3β inhibition or that DADS inhibits GSK-3β through a direct interaction.

DADS inhibition of NF-κB and COX-2 was GSK-3β dependent

To determine whether DADS suppression of NF-κB activity is dependent on GSK-3β inhibition, we treated SW480 cells with DADS together with a GSK-3β activator, LY294002. When cells were treated both with LY294002 and DADS, the decreased phosphorylation and nuclear translocation of P65-NF-κB, as well as the COX-2 inhibition, previously observed with DADS only treatment, was attenuated (Fig. 4A). These results suggested that DADS-mediated NF-κB inhibition is GSK-3β-dependent. To evaluate whether the GSK-3β-dependent inhibition of NF-κB correlated with a decrease in tumorigenicity, we employed the use of a soft agar assay to monitor anchorage-independent three-dimensional colony formation. This method can deliver results that are comparable with those obtained when injecting tumorigenic cells into nude mice (41). Treatment with 5 μmol/L DADS was sufficient to prevent the ability of SW480 cells to form colonies in soft agar. The simultaneous treatment with a GSK-3β activator LY294002 (10 μmol/L) and DADS (5 μmol/L) abolished antitumorigenic effects of DADS (Fig. 4B). These results show that
DADS prevents tumorigenicity in vitro, which appears to be dependent of GSK-3β inhibition and likely to be contributed to the inhibition of the NF-κB pathway.

**DADS inhibited GSK-3β and NF-κB in vivo**

To determine whether the effects of DADS on GSK-3β inhibition and NF-κB localization seen in SW480 cells can be translated in vivo, we measured using IHC phosphorylated GSK-3 (α/β) at Serine (21/9) and nuclear localization of phosphorylated NF-κB (P65) Serine 536 in the colons of AOM/DSS-treated mice. Similar to the SW480 cells, mice that consumed a diet supplemented with 85 ppm of DADS had a significantly higher level of inactivated GSK-3 (α/β) compared with mice on the control diet and a significantly lower nuclear localization of phosphorylated NF-κB (P65; Fig. 5A and B). Furthermore, the changes correlated with an 86.2% decrease in Ki-67 expression, a marker of cellular proliferation (Fig. 5C). These results further confirm that DADS inhibits GSK-3β and suppresses the activation and nuclear localization of NF-κB, which may in part contribute to the chemopreventive activity of DADS.

**Discussion**

Epidemiologic evidence has shown an association between garlic intake and decreased risk of developing colorectal cancer (42, 43). Likewise, considerable experimental evidence has shown that garlic sulfur-containing constituents, such as diallyl disulfide (DADS), can reduce the growth of colon tumor cells in animals (44, 45). To our knowledge, the underlying mechanism explaining the antitumorigenic effects of DADS has yet to be elucidated. In the current study, we examined the hypothesis that DADS exerts its protective effects against colorectal tumors by suppressing inflammation, utilizing the colitis-induced colorectal cancer AOM/DSS mouse model. Our results indicated that dietary supplementation with 42 and 85 ppm DADS (HED of 30 and 60 mg/daily, respectively) for 32 days resulted in a significantly shortened recovery time from the DSS-induced inflammatory insult and significantly reduced number and size of colorectal tumors in AOM/DSS-treated mice. In vitro studies using the human CRC SW480 cell line revealed that the tumor-suppressive effect of DADS may, in part, be mediated by GSK-3β–dependent NF-κB inhibition.

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**Figure 5.**

DADS induced GSK-3α/β phosphorylation at S21/9, inhibited nuclear localization NF-κB (P65) phosphorylated at S536, and Ki-67 expression in vivo. A-C, immunohistochemical analysis p-S21/9 GSK-3α/β (1:100), p-S536-NF-κB (P65; 1:100), and Ki-67 (1:100) in colonic epithelium at day 52 from mice from AOM/DSS control group or 85 ppm DADS group. A, DADS induced inhibition of GSK-3α/β as shown by increased phosphorylation at S21/9 with both cytoplasmic and nuclear localization. B, DADS reduced expression of p-S536-NF-κB (P65) in nucleus. C, DADS inhibited proliferation as shown by staining with Ki-67. Scale bar, 100 μm. Right, quantitation of staining using Aperio Scanscope nuclear staining program. **.**, P < 0.01 indicates a significant difference compared with control group as determined by t test.

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Constitutive NF-κB activation in inflammatory bowel disease is associated with an increased risk of developing colorectal cancer (15). The NF-κB heterodimeric REL-A (P65)-p50 complex is sequestered in the cytoplasm under nonstimulated conditions by IκB. Proinflammatory cytokines within the tumor microenvironment, such as TNF1 and IL1, induce phosphorylation of IκB by IKK complexes, which allows the P65:p50 heterodimer to be released and translocate to the nucleus where they activate various target genes (16). IκKs and IκB inhibitors are the mainstay of targeting NF-κB activity by inhibiting IKK activity, increasing concentrations of DADS had no effect on either IκK or IκB, indicating the upstream canonical pathway is not involved in DADS inhibition of NF-κB. There was, however, a decrease in phosphorylated P65 (NF-κB-P65S536) in the nucleus with a corresponding increase in the cytoplasm, suggesting that the suppressive effect of DADS on NF-κB activity is likely due to its ability to inhibit the nuclear translocation of the phosphorylated P65. This finding was consistent with the decrease in NF-κB phosphorylation in colon tissue of DADS-fed mice.

GSK-3 is a serine/threonine kinase that has diverse cellular functions and numerous substrates. In our study, DADS, a major anti-inflammatory component in garlic, inactivated GSK-3 (a/b) by blocking its phosphorylation of serine residue 21/95 and this event was determined to inhibit NF-κB nuclear translocation. As mentioned previously, NF-κB transcription family members associate as homodimers and heterodimers complexes, of which the RELA (P65)/p50 is the most abundant (38). The p50 protein is produced from constitutive processing of p105 through ubiquitin-mediated proteolysis, a process mediated by IKK (47). In addition, the precursor protein p105 is responsible for the cytoplasmic retention of NF-κB heterodimers (48). Interestingly, GSK-3 has been shown to phosphorylate p105 in vitro, accelerating p105 proteolysis to p50 (39). Furthermore, downregulation of GSK-3 in fibroblasts was shown to inhibit NF-κB induction (20). Thus, it is possible that DADS inhibition of GSK-3 is disrupting the distribution of p105 and p50, resulting in reduced nuclear expression and translocation of the active RELA P65/p50 NF-κB heterodimer. Our notion that DADS inhibition of GSK-3 is responsible for the observed anti-inflammatory effects, including the inhibition of NF-κB is further supported by an in vivo study that demonstrated that administration of GSK-3 inhibitors could effectively downregulate NF-κB activity and significantly attenuate TNBS-induced colitis in rats (49).

In conclusion, we have provided evidence that garlic constituents, DADS interfere with nuclear translocation of pro-inflammatory transcription factor NF-κB and the expression of tumorigenic enzyme COX2 via GSK-3 (a/b) inactivation. Future studies will be needed to determine the exact mechanism of DADS-induced GSK-3 (a/b) inactivation. In addition, there are over 30 known organosulfur compounds in garlic (50) that should also be explored further. The importance of dietary components including DADS and metabolic enzymes such as GSK-3 (a/b) in maintaining homeostasis of intracellular microenvironment to prevent perpetual inflammatory pathways therefore cannot be overemphasized.

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