Carboxyamidotriazole Ameliorates Experimental Colitis by Inhibition of Cytokine Production, Nuclear Factor-κB Activation, and Colonic Fibrosis

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ABSTRACT

Carboxyamidotriazole (CAI) has been reported to suppress the production of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β and be effective in rats with adjuvant arthritis. The aim of this study was to investigate the role of CAI in inflammatory bowel disease (IBD). We assessed the effect of CAI in dextran sodium sulfate-induced colitis. Inflammation was scored histologically, and potential mediators of IBD were assessed by immunohistochemical and molecular biochemical approaches. CAI-treated colitis animals revealed much fewer signs of colitis with significantly decreased macroscopic and microscopic scores than vehicle-treated animals. CAI inhibited the production of TNF-α, IL-1β, and IL-6 in serum, supernatant of peritoneal macrophages, and lamina propria. CAI also decreased the expression of intercellular adhesion molecule-1 in colonic tissues. Furthermore, CAI prevented nuclear factor-κB (NF-κB) activation and inhibitor of nuclear factor-κB phosphorylation and degradation. In addition, CAI showed a beneficial effect on colonic fibrosis, possibly by reducing the production of the fibrogenic cytokine transforming growth factor-β. The results support that CAI administration is effective in ameliorating experimental colitis and preventing colonic fibrosis. The inhibition of proinflammatory cytokines and adhesion molecules and suppression of NF-κB activation seem to contribute to this effect.

Introduction

Ulcerative colitis (UC) and Crohn’s disease (CD) are the two major forms of inflammatory bowel disease (IBD) and are characterized by nonspecific inflammation and intestinal tissue damage. Although the etiologies of both diseases remain poorly understood, clinical and experimental evidence increasingly suggest that the basis of IBD is multifactorial, involving disordered activation of lamina propria macrophages, lymphocytes, and neutrophils derived from the circulatory system (Mahida, 2000). The high amount of proinflammatory cytokines, particularly tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, produced by these cells plays a major role in the initiation and perpetuation of intestinal inflammation, which has been confirmed in patients with IBD and various animal models resembling IBD, including the dextran sodium sulfate (DSS)-induced colitis model (Elson et al., 1995; Fiocchi, 1998; Strober and Fuss, 2011). In addition, many adhesion molecules are responsible for the local recruitment of inflammatory cells, including E-selectin, which mediates the initial rolling of leukocytes on vascular endothelium, and intercellular adhesion molecule-1 (ICAM-1), which interacts with integrins expressed on leukocytes and results in firm adhesion in order for gut inflammation to proceed in IBD. Furthermore, it was found that nuclear factor-κB (NF-κB), the key regulator of inflammatory cytokine gene transcription, seems to be a central mediator of immune and inflammatory responses (Bonizzi and Karin, 2004). In...
resting cells, NF-κB is sequestered in the cytoplasm as an inactive complex bound to inhibitor proteins termed inhibitor of nuclear factor-κB (IκB) (e.g., IκBα, IκBβ, IκBγ, and IκBε). In a canonical activating pathway induced by various NF-κB stimuli, such as proinflammatory cytokines (e.g., TNF-α and IL-1β), lipopolysaccharide (LPS), and phorbol ester, at least two of the IκB proteins (IκBα and IκBβ) are phosphorylated rapidly by the IκB kinase (IKK) complex and degraded, allowing NF-κB to translocate into the nucleus and in turn activate numerous genes, such as cytokines (TNF-α, IL-1, IL-6, IL-8, and IL-12), chemokines (monocyte chemoattractant protein-1), adhesion molecules (ICAM-1), or vascular cell adhesion molecule-1 (VCAM-1) (Neurath et al., 1998; Oeckinghaus et al., 2011). This positive feedback regulation between NF-κB and the inflammatory mediators contributes to the crucial initial steps of inflammation as well as its perpetuation. The activated form of NF-κB is a heterodimer, which usually consists of two proteins, a 65-kDa subunit (p65 or relA) and a 50-kDa subunit (p50). In particular, the p65 subunit was highly activated in epithelial cells and lamina propria macrophages from patients with active UC and CD (Rogler et al., 1998).

Carboxamidotriazole (CAI), a well-tolerated antiangiogenic agent, has been shown to inhibit proliferation and invasive behavior of a wide array of human cancer cell types and endothelial cells in vitro and in vivo (Kohn et al., 1992, 1994, 1995). We discovered recently that CAI has considerable anti-inflammatory potency, and it has been used effectively in the treatment of rat adjuvant-induced arthritis. Although the mechanisms of the anti-inflammatory action of CAI are not completely understood, results from studies indicated that CAI is capable of down-regulating the levels of proinflammatory cytokines (e.g., TNF-α and IL-1β) at the site of inflammation and in serum (Gou et al., 2008). Another noticeable finding is that the pretreatment of human T cells with CAI inhibits the nuclear accumulation of c-Rel and p65, causing selective repression of NF-κB DNA binding and near-complete inhibition of calcium-regulated mitogen-induced transcription from the HIV long-terminal repeat (Yasui et al., 1997).

On the basis of these observations, we hypothesized a role for CAI in experimental colitis. We speculated that the down-regulating effect of CAI on proinflammatory cytokines and its antiangiogenic property could inhibit intestinal inflammation and colonic fibrosis, respectively, and thereby be helpful to prevent and treat IBD. As presented below, the administration of CAI ameliorated the outcome of DSS-induced colitis in mice.

Materials and Methods

Materials. CAI was synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences. Polyethylene glycol 400 (PEG 400) was provided by Beijing Chemical Reagents Company (Beijing, China). Sulfasalazine (SASP) was from Shanghai Sanwei Pharmacy Co. Ltd. (Shanghai, China), DSS (36,000–50,000 mol. wt.) was purchased from International Laboratory (San Bruno, CA). Enzyme-linked immunosorbent assay kits for TNF-α, IL-1β, IL-6, and transforming growth factor (TGF)-β1 were from R&D Systems (Minneapolis, MN). The hydroxyproline assay kit was from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Primary antibodies against TNF-α, p65, ICAM-1, p-IκBα, IκBα, and β-actin and peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

DSS-Induced Colitis and Design of Treatment. In the experiment, 8- to 10-week-old, male, C57BL/6 mice (Beijing Vital River Laboratory Animal Co., Ltd., Beijing, China) were assigned randomly to one of five groups of 15 animals each on day 0. Colitis was induced in four groups of mice by adding 3% DSS to their drinking water and allowing them to drink ad libitum (Okayasu et al., 1990). Normal mice not submitted to any intervention constituted the control group. The colitis groups intra-gastrically received one of the following treatment regimes daily: 1% Sodium Carboxy Methyl Cellulose (model group), PEG 400 mg/kg CAI, and 300 mg/kg SASP. The period of administration was from day 8 to day 17 during 17 days of DSS exposure. After 10 days of specific treatment, mice were euthanized.

Macroscopic Assessment of Colonic Damage. Animals were weighed daily and inspected for diarrhea and rectal bleeding (based on guaiac reaction, hemoccult) using a score from 0 to 4. For stool consistency, 0 was given for well formed pellets, 2 for purty and semiformal stools that did not stick to the anus, and 4 for liquid stools that did stick to the anus. Bleeding was scored 0 for no blood in hemoccult, 2 for positive hemoccult, and 4 for gross bleeding. After the animals were sacrificed, the colon was removed, opened longitudinally, and washed with phosphate-buffered saline (PBS). Macroscopic damage was assessed by the scoring system of Wallace and Keenan (1990), which takes into account the area of inflammation and the presence or absence of ulcers plus the adhesion score (0 for no adhesion, 1 for minimal adhesion, and 2 for adhesions involving several bowel loops) and colon structure score (0 for no stricture, 2 for mild stricture, and 3 for severe stricture with proximal dilatation). The criteria for assessing the ulceration score were as follows: 0, no ulcer, no inflammation; 1, no ulcer, local hyperemia; 2, ulceration without significant inflammation; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 0.5 cm.

Microscopic Assessment of Colonic Damage. For histological analysis, tissue samples were fixed in 10% formalin in PBS overnight. Four-micrometer specimens were subjected to hematoxylin and eosin staining. Randomly selected fields in sections (n = 45) from at least six mice of each group were inspected under a light microscope and graded. For stool collection on the last day of the experiment. Peritoneal macrophages were isolated from colitis mice and challenged with LPS for 18 h as described below. To prepare colon tissue homogenates, the colon of each animal was removed, opened longitudinally, and cleaned of fecal material. Tissues were homogenized subsequently in ice-cold buffer [0.1 M potassium phosphate (pH 7.8), 1 mM phenylmethyl-sulfonyl fluoride, and 10 μg/ml aprotinin] with a tissue homogenizer and centrifuged to remove debris. These samples of serum, supernatants of peritoneal macrophages, and colon homogenates were stored at −80°C until further analysis. TNF-α, IL-1β, and IL-6 levels in all of the samples as well as TGF-β1 protein in colon tissues and supernatants of peritoneal macrophages were measured using specific mouse enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions. The sensitivities of the assays for TNF-α, IL-1β, IL-6, and TGF-β1 were 5.1, 7, 4, and 5 pg/ml, respectively.

Peritoneal Macrophage Isolation and Culture. To study the in vitro effect of CAI on cytokine production, macrophages were isolated from the peritoneal cavity of male C57BL/6 mice (18–22 g; 8–10 weeks old). In brief, 6% starch broth was injected into the
peritoneal cavity for 3 days, 1 ml/day per animal. Elicited peritoneal macrophages were isolated by flushing the peritoneum with 3 ml of ice-cold PBS. Red blood cells were lysed, and the remaining cells were washed extensively using ice-cold PBS. Peritoneal macrophages were seeded into a 24-well tissue culture plate at a final concentration of 1 × 10^6 cells per milliliter. After 4 h, the medium was removed and replaced with fresh serum-free medium containing 1 μg/ml LPS and various concentrations of CAI (5, 10, 20, and 30 μM). Supernatants were collected after 18 h for cytokine level determination (Alleva et al., 2002).

**Immunohistochemical Detection of TNF-α, p65, and ICAM-1 in Colon Tissues.** The paraffin-embedded colonic sections were deparaffinized for immunohistochemical detection of cells positively stained for TNF-α, p65, and ICAM-1. Endogenous peroxidase activity was blocked using 3% H_2O_2 for 10 min, followed by incubation with nonspecific staining blocking reagent (Dako, Carpinteria, CA). Hematoxylin was used for nuclear counterstaining. DAB Peroxidase Substrate kit (Vector Laboratories, Burlingame, CA) was used for nuclear counterstaining.

Randomly selected fields in sections (n = 45) from at least six mice from each group were inspected and graded. Immunohistochemical score was calculated based on the sum of a proportion score (percentage of positive-stained cells: 0, none; 1, <30%; 2, 30–70%; 3, >70%) and an intensity score (0, none; 1, weak; 2, intermediate; 3, strong). The numerical rating score was as follows: 0, negative; 2–3, weak positive; 4, positive; 5–6, strong positive (Allred et al., 1998).

**Western Blot Analysis.** Western blot analysis was performed on lysates of peritoneal macrophages obtained from naive (control), Sodium Carboxyl Methyl Cellulose- (model group), PEG 400-, CAI, and SASP-treated mice (four mice per group). Cells were lysed on ice with a solution containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 20 mM β-glycerol phosphate, 50 mM NaF, 1 mM dithiothreitol, 1% Nonidet P-40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was cleared by centrifugation at 14,000g for 15 min. Protein content in the supernatant fraction was determined by the method of Bradford. Lysate containing 40 μg of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis, and the proteins were transferred onto a polyvinylidene fluoride membrane. After having been blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween 20, the membrane was incubated with the desired primary antibody for 2 h at the following dilutions: p-IκBα (1:1000), IκBα (1:1000), and β-actin (1:500). The membrane was incubated subsequently with the appropriate secondary antibody, and the immunoreactive protein bands were visualized using an enhanced chemiluminescence kit (Millipore Corporation, Billerica, MA) according to the manufacturer’s instructions. All of the proteins were detected by enhanced chemiluminescence-based autoradiography as recommended by the manufacturer (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The density ratio of a specific band over β-actin was determined with a densitometer.

**Assessment of Colonic Fibrosis.** For colonic fibrosis assessment, the paraffin-embedded colonic sections were stained with a Masson Trichrome Stain kit (Maxim IHC World, Fuzhou, China). Collagen area was defined as the distinct blue region and distinguished from muscle, blood, and inflammatory cells. Cell nuclei were stained dark brown, and the background was stained red (Jones et al., 2008).

**Collagen Determination.** The whole frozen colon tissues were ground and hydrolyzed with 6 N HCl for 5 h at 100°C. Aliquots of the hydrolysate were processed for the quantification of hydroxyproline as an index of collagen content using a modified hydroxyproline assay kit based on the method described previously (Mourelle et al., 1996).

**Statistical Analysis.** The results are expressed as the mean ± S.E. for in vivo data or mean ± S.D. for in vitro data, where n represents the number of experiments or animals. Data were analyzed using SPSS 11.0 software (SPSS Inc., Chicago, IL). Differences between the groups were compared using one-way analysis of variance followed by Dunnett’s test. P values less than 0.05 (P < 0.05) were considered as indicative of significance.

**Results**

CAI Decreases the Susceptibility of Mice to DSS-Induced Colitis. During the course of DSS treatment in the

![Fig. 1](image-url)
study, there were apparent differences in the macroscopic and microscopic appearance of the colons of vehicle-treated colitis mice and drug-treated colitis mice. Model or PEG 400-treated colitis mice exhibited profound body weight loss, diarrhea, intestinal bleeding, and mucosal ulceration, whereas CAI- or the reference drug SASP-treated mice had less body weight loss and demonstrated relatively poor diarrhea and bleeding scores (Fig. 1). In the experiment, CAI-treated mice recovered quickly and significantly from the day after administration (day 9) and showed a growing trend of improvement (Fig. 1, A–C). After 10 days of specific treatment, the beneficial effect of CAI was confirmed further by satisfied macroscopic appearance, such as reduced colon hyperemia, inconspicuous thickening of the intestinal wall, and less ulceration, with a markedly reduced macroscopic score (2.50 ± 0.72 in CAI-treated mice versus 5.67 ± 0.35 in PEG 400-treated mice, P < 0.001, n = 15). In addition, histological analysis demonstrated that CAI prevented and ameliorated DSS-induced colonic injury with an effect comparable to that of the reference drug SASP. Samples from the model or PEG 400-treated colitis group showed gross ulceration accompanying massive infiltration of leukocytes into the mucosa and congestion and edema of the submucosa with loss of entire crypts and surface epithelium, whereas CAI-treated mice presented less cellular infiltration and no ulceration, with colonic architecture preserved, leading to a much lower histologic score compared with that of the vehicle-treated colitis group (0.62 ± 0.12 versus 3.69 ± 0.09, P < 0.001, n = 15) (Fig. 2).

### CAI Reduces Proinflammatory Cytokine Production.
It has been well documented that proinflammatory cytokines play critical roles in CD pathogenesis, so we evaluated the effect of CAI on the production of inflammatory cytokines involved in DSS-induced colitis, including TNF-α, IL-1β, and IL-6. As shown in Table 1, significantly higher levels of TNF-α, IL-1β, and IL-6 were found in the serum, supernatants of peritoneal macrophages, and colon tissues from colitis mice compared with those of normal controls. CAI treatment strikingly reduced the production of these proinflammatory cytokines compared with those obtained from model and PEG 400-treated colitis groups, except that the concentration of IL-1β in serum from CAI-treated colitis

![Fig. 2. Histological assessment of DSS-induced colitis. A–E, representative hematoxylin and eosin staining sections of the transverse colon in control mice without DSS (A), model colitis mice (B), PEG 400-treated colitis mice (C), CAI-treated colitis mice (D), or SASP-treated colitis mice (E) (left, original magnification, 50×; right, original magnification, 200×). Note the regional mucosal destruction in model or vehicle-treated mice (B and C), including goblet cell loss, crypt loss, significant cellular infiltration in the lamina propria, and submucosal edema. In contrast, colonic architecture is preserved and cellular infiltration is reduced markedly in CAI- or SASP-treated mice (D and E). F, histologic score. Data represent mean values ± S.E. of n = 6 mice in each group. ***, P < 0.001 compared with PEG 400-treated mice. ###, P < 0.001 compared with model mice.](image-url)
mice decreased slightly but not enough to make a significant prediction (Table 1). Furthermore, in our experiment, the expression of TNF-α in colonic mucosa was detected by immunohistochemistry. There was no detectable TNF-α in normal colonic tissues corresponding to the lowest immunohistological score of 0.62 ± 0.16 (Fig. 3A). But under intestinal inflammation, a mass of TNF-α-positive cells was scattered within the inflamed colonic mucosa of DSS-induced colitis, resulting in much higher immunohistological scores of 5.88 ± 0.34 and 5.93 ± 0.38, respectively, in the model and PEG 400-treated groups (Fig. 3, B and C). CAI treatment significantly inhibited TNF-α expression in colonic mucosa, accordingly presenting a marked reduction in TNF-α immunohistological score (2.29 ± 0.18, P < 0.001 compared with PEG 400-treated mice) (Fig. 3D).

CAI Decreases the Expression of ICAM-1 in Colonic Tissues. ICAM-1 expressed by endothelial cells plays an important role in trafficking leukocytes into the areas of inflammation and IBD pathogenesis. To further explore the effect of CAI, we compared ICAM-1 expression in normal and colitis mice with or without drug treatment. Microscopic examination of the samples taken from normal control mice showed very weak constitutive expression of ICAM-1 in the lamina propria of colonic mucosa with the lowest immunohistological score of 0.81 ± 0.10 (Fig. 4A). In the model and PEG 400-treated colitis groups, a well marked ICAM-1 up-regulation was observed, with the immunohistological scores increased to 6.35 ± 0.29 and 6.02 ± 0.38, respectively. The enhancement of immunohistochemical staining was especially evident in the inflamed lamina propria and submucosa (Fig. 4, B and C). Although the CAI-treated colitis mice displayed much weaker staining, demonstrating far fewer immunopositive cells, leading to a significantly decreased immunohistological score of 1.95 ± 0.09 (P < 0.01, compared with the PEG 400-treated colitis group) (Fig. 4D).

CAI Down-Regulates NF-κB Activation by DSS. NF-κB is an important regulator of TNF-α and IL-1β gene expression. Because in DSS-induced colitis these cytokines are up-regulated and profoundly decreased by 20 mg/day CAI treatment as we have demonstrated (Table 1), we were interested in determining the activation state of NF-κB in our study animals. NF-κB activity is controlled by the steady-state concentration of IκBα; therefore, we analyzed both NF-κB p65 subunit and IκBα expression as well as its phosphorylation status in colon biopsy and macrophage protein samples. Tissue sections of colons from normal mice demonstrated low levels of NF-κB p65 expression mainly in the cytoplasm of vascular endothelial cells. The majority of tissue sections from model or PEG 400-treated colitis mice showed overexpression of NF-κB p65 in the submucosa of inflamed colon tissues, particularly in the nuclei of macrophages and epithelial cells as well as vascular endothelial cells. In con-

### Table 1

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<tr>
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<th>Control</th>
<th>Model</th>
<th>PEG 400</th>
<th>CAI</th>
<th>SASP</th>
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<tr>
<td>Serum, pg/ml</td>
<td>19.30 ± 0.38</td>
<td>81.32 ± 7.07</td>
<td>79.16 ± 10.13</td>
<td>26.32 ± 7.26*</td>
<td>29.97 ± 0.57*</td>
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<td>TNF-α</td>
<td>9.80 ± 1.40</td>
<td>150.63 ± 8.87</td>
<td>144.25 ± 11.50</td>
<td>115.12 ± 10.05</td>
<td>120.04 ± 11.06</td>
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<tr>
<td>IL-1β</td>
<td>37.26 ± 1.25</td>
<td>180.84 ± 9.84</td>
<td>182.86 ± 4.98</td>
<td>134.42 ± 3.42*</td>
<td>160.76 ± 5.06</td>
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<tr>
<td>Colon, pg/mg protein</td>
<td>49.36 ± 22.11</td>
<td>169.34 ± 10.98</td>
<td>176.90 ± 6.18</td>
<td>79.60 ± 2.37***</td>
<td>137.71 ± 0.78**</td>
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<tr>
<td>TNF-α</td>
<td>3.15 ± 0.16</td>
<td>41.51 ± 0.49</td>
<td>43.47 ± 6.05</td>
<td>10.89 ± 1.61***</td>
<td>14.51 ± 0.51**</td>
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<tr>
<td>IL-1β</td>
<td>7.34 ± 4.81</td>
<td>36.99 ± 6.11</td>
<td>31.43 ± 0.63</td>
<td>11.21 ± 0.35**</td>
<td>16.89 ± 0.32**</td>
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<td>Macrophage, pg/ml</td>
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<tr>
<td>TNF-α</td>
<td>263.08 ± 9.17</td>
<td>257.14 ± 8.03</td>
<td>50.38 ± 6.88***</td>
<td>64.70 ± 2.29**</td>
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<td>IL-1β</td>
<td>210.44 ± 10.38</td>
<td>217.78 ± 7.52</td>
<td>189.40 ± 1.96**</td>
<td>174.69 ± 17.06*</td>
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<td>IL-6</td>
<td>178.26 ± 13.50</td>
<td>167.69 ± 28.44</td>
<td>49.45 ± 1.77**</td>
<td>12.26 ± 11.25**</td>
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* P < 0.05; ** P < 0.01 compared with PEG 400-treated mice. # P < 0.05; ## P < 0.01 compared with model mice.
NF-κB p65 staining was decreased significantly in colon tissues from CAI- or SASP-treated mice (Fig. 5, A–E). The p65 immunohistological scores in each group were 1.05 ± 0.39 (control), 6.22 ± 0.43 (model), 5.99 ± 0.29 (PEG 400), 2.05 ± 0.28 (CAI), and 3.99 ± 0.34 (SASP), respectively. As to the activation of NF-κB in macrophages from mice with various treatments, we determined the levels of IκBα, an endogenous inhibitor of NF-κB, as well as its phosphorylated form p-IκBα in the cytosolic extracts by Western blot analysis. As shown in Fig. 5F, the levels of p-IκBα were increased, and the levels of IκBα were decreased in the colitis model group and PEG 400-treated group. With CAI or SASP treatment, DSS-induced degradation of IκBα was suppressed significantly (Fig. 5F). These findings indicated that CAI inhibits the activation of NF-κB in vivo during inflammation.

CAI Prevents Colonic Fibrosis. Colonic tissues stained with Masson’s Trichrome exhibited an obvious increase in transmural collagen deposition, indicating significant fibrosis in model colitis mice and DSS-PEG 400 mice. The serosa as well as the subserosal region (between the serosa and muscularis propria) of the model or PEG 400-treated mice appeared thicker than that in the control colon (from normal mice without any manipulation) in association with increased cellularity of this region after DSS exposure for 17 days (Fig. 6, B and C). However, CAI- or SASP-treated mice had little evidence of fibrosis, with the majority of collagen deposition confined to the submucosa, as is found in the control colon (Fig. 6, A, D, and E). In addition, there was a significant difference in hydroxyproline content per milligram of colonic tissue that acts as a quantitative index of collagen content between PEG 400 and CAI groups (Fig. 6F). Mean colonic collagen content of the model or PEG 400-treated mice increased by 53.5 and 41.9%, respectively, compared with that of control mice. However, mice with colitis receiving CAI treatment showed roughly the same collagen content as that of control mice and markedly lower than that of PEG 400-treated mice (P < 0.05). The collagen content of SASP-treated mice tended to be lower, but not significant, than that
of model mice. Furthermore, DSS-induced colitis in the model or PEG 400 group was associated with a significant increase in TGF-β1 levels in colon tissue homogenates (207.78 ± 4.58 and 221.42 ± 4.54 pg/mg protein, respectively) compared with that of the control group (20.48 ± 3.77 pg/mg protein). Administration of CAI (20 mg/kg) to colitis mice resulted in significantly decreased TGF-β1 (105.33 ± 4.65 pg/mg protein, \( P < 0.001 \), compared with that of the PEG 400-treated colitis group). A weaker reduction trend in TGF-β1 protein level also was noted in colitis mice with SASP (300 mg/kg) treatment (189.27 ± 3.92 pg/mg protein, \( P < 0.05 \)) (Fig. 6G). To further verify the effect of CAI on TGF-β1 production, macrophages isolated from the peritoneal cavity of healthy C57BL/6 mice were stimulated with 1 μg/ml LPS and incubated with various concentrations of CAI for 18 h. CAI decreased the production of TGF-β1 in a dose-dependent manner. Significant inhibition was seen when cells were exposed to CAI at concentrations of 5 μM or above (Fig. 6H). The results supported a direct suppressive effect of CAI on TGF-β1 production.

**Discussion**

CD and UC are idiopathic inflammatory bowel disorders driven by many genetic, immunologic, and environmental factors. Over the past few decades, enormous progress has been made in understanding the pathogenesis of these diseases and developing new treatment strategies. The reports that TNF-α levels elevate in serum of CD patients, stool of IBD patients, and culture supernatants of colonic biopsies from IBD patients and that TNF-α mRNA expression was enhanced in colonic mucosa from UC or CD patients led us to consider TNF-α as a key mediator for IBD pathogenesis and maintenance and subsequently as a potential key therapeutic target (Murch et al., 1991; Braegger et al., 1992; Cappello et al., 1992; Reimund et al., 1996). This hypothesis has been reinforced by animal studies showing that anti-TNF-α antibody therapy significantly improves experimental colitis. Moreover, the use of an antibody specific for TNF-α (infliximab, etanercept, and adalimumab) has proven already to be a highly effective treatment in CD, achieving positive effects in 60% of patients (Reimund et al., 2007; Rutgeerts et al., 2009). However, this costly treatment is administered via repeated injections and regrettably with primary nonresponse or loss of response in clinical use from the formation of anti-drug antibodies or immune and nonimmune clearance of anti-TNF-α biologicals (Allez et al., 2010). Hence, there is a need for cheaper, orally available, nonbiological treatments that reduce the production of TNF-α and other inflammatory mediators.

This study provides the first in vivo evidence for the beneficial effect of CAI in IBD. We induced a slowly progressive colitis in C57BL/6 mice with 3% DSS (mol. wt. 36,000–50,000) that is characterized by ulceration, epithelial damage, mucosal or transmural inflammatory infiltrates, and lymphoid hyperplasia. Our results suggested that CAI reduced the release of inflammatory mediators, macroscopic and histological damage scores, and colonic collagen content in the experimental mouse model of colitis. Daily administration of CAI significantly inhibited the progression of colitis, showing a protective effect equal to or even greater than

![Fig. 5. Immunohistochemical detection of NF-κB p65 expression in colons from control mice without DSS induction (A), model colitis mice (B), PEG 400-treated colitis mice (C), CAI-treated colitis mice (D), and SASP-treated colitis mice (E) (original magnification, 400×). F, CAI blockade of IκBα degradation and phosphorylation in peritoneal macrophage cells. Western blot analysis was performed with anti-IκBα and anti-\(p\)-IκBα bodies. Densitometric analysis was normalized to actin.](image-url)
that of SASP. It is widely accepted that the driver of tissue damage arising in IBD is the excessive activation of adaptive immunity, which is affected by a combination of resident and recruited cell populations. After an inflammatory signal, circulating macrophages migrate to the intestinal mucosa, and these cells are activated by proinflammatory cytokines secreted by CD4⁺ T helper cells, resulting in greater production of IL-1, IL-6, and TNF-α. The biologic effects of these proinflammatory cytokines result in the amplification of immunologic and inflammatory processes. For example, TNF-α activates endothelial cells and can induce the synthesis and secretion of IL-1 and IL-6. IL-1 stimulates acute-phase protein synthesis and initiates T cell activation events. Proinflammatory cytokines and chemokines increase the expression of endothelial cell adhesion molecules, which in turn enhance the influx of monocytes, granulocytes, and lymphocytes into areas of chronic inflammation. Proinflammatory cytokines therefore are of central importance in the initiation and perpetuation of intestinal inflammation in UC and CD (Baumgart and Carding, 2007; Xavier and Podolsky, 2007).

In this study, TNF-α, IL-1β, and IL-6 levels in serum, supernatant of peritoneal macrophages, and colon tissues were reduced significantly and correlated with inflammatory scores. In fact, we have reported that the antagonistic effect of CAI against the production of TNF-α and IL-1β might account for its beneficial effects on adjuvant arthritis in rats (Guo et al., 2008). Hence, we suppose the blockade of proinflammatory cytokine production makes CAI an interesting candidate for the treatment of other inflammatory diseases besides IBD and rheumatoid arthritis in which TNF-α, IL-1β, or IL-6 plays an important role, such as ankylosing spondylitis and psoriasis.

Proinflammatory cytokines are known to up-regulate a spectrum of cell adhesion molecules, including ICAM-1, VCAM-1, selectins, and integrins. In the study of human IBD, ICAM-1-positive inflammatory infiltrates and venules were demonstrated to increase in parallel to the degree of inflammation, whereas VCAM-1 was not enhanced significantly in the inflamed mucosa (Sans et al., 1999). ICAM-1, a member of the immunoglobulin superfamily, is an inducible transmembrane glycoprotein that is constitutively expressed at low levels on vascular endothelial cells and a subset of...
leukocytes and will play a central role in the recruitment and retention of leukocytes in inflammatory foci and further mediate enhanced leukocyte adhesion and interaction (Long, 2011). In the present study, the expression of ICAM-1 in colon samples was determined by immunohistochemistry, and the results showed that DSS-induced mouse colon mucosa displayed a higher level of ICAM-1 protein expression that was closely related to the degree of the mucosal lesions and inflammation. However, the CAI-treated colitis mice displayed significantly weak staining, demonstrating far fewer immunopositive cells. In light of the fact that CAI is a recognized antiangiogenic agent, we think the reduction of ICAM-1 staining may result partly from the down-regulation of endothelial cell proliferation. Then, a reduced interaction with leukocytes and diminished inflammation activity after CAI treatment will come when conditions are ripe.

As is well known, activation of NF-κB plays an important role in intestinal inflammation (Neurath et al., 1996; Kaser et al., 2010), which is confirmed in the present study. NF-κB is complexed in the cytoplasm, where it is inactive, through an interaction of the p65 or c-Rel subunit with inhibitory proteins termed IκB (e.g., IκBα, IκBβ, and IκB) (Neurath et al., 1998). In response to a variety of extracellular stimuli, such as proinflammatory cytokines (e.g., IL-1 and TNF-α), LPS, and phorbol ester, the IKK complex is activated and phosphorylates IκB proteins on N-terminal serines, leading to their ubiquitination and subsequent proteasome-dependent degradation. After IκBα is degraded, NF-κB is activated, allowing its translocation into the nucleus and the activation of numerous genes, including adhesion molecules (ICAM-1), VCAM-1, chemokines, or cytokines (e.g., TNF-α, IL-1, IL-6, IL-8, and IL-12) (Ghosh and Karin, 2002; Oeckinghaus et al., 2011). Thus, NF-κB is recognized as a central “switch” in the crucial initial steps of inflammation as well as in its perpetuation. In accordance with the idea, we observed an up-regulation of NF-κB activity manifested by overexpressed p65 protein in inflamed colonic tissue and increased p-IκBα levels in cytosolic extracts of peritoneal macrophages from mice fed with DSS compared with those of normal animals. Immunohistochemical analysis showed decreased expression of NF-κB p65 by the administration of CAI, and Western blot analysis further confirmed that the degradation of IκBα was also inhibited by CAI. Although we could not specify the exact target of CAI, these data and aforementioned cytokine- and adhesion molecule-related results suggest that CAI works on a cytokine-integrating signal pathway, and the direct inhibition of IκBα degradation and NF-κB activation appears to be a key mechanism for further inhibiting the important proinflammatory cytokine feedback loop (e.g., TNF-α → IKK → IκB → NF-κB → TNF-α) in IBD (Fig. 7).

Our study also demonstrated that CAI effectively improved chronic colonic inflammatory lesions, with a marked reduction of colonic collagen deposition compared with those of model and PEG 400-treated groups on day 17 after the induction of colitis. This improvement of fibrosis could be explained by a decrease in the severity of mucosal inflammation resulting from the reduction of proinflammatory cytokine secretion after CAI treatment. Besides, a marked reduction of TGF-β1 levels in colonic tissues that might be an accompanied effect after colitis improvement or was a result of direct inhibition by CAI contributed to the specific effect of CAI against fibrosis. TGF-β1 is a cytokine that plays an important role in regulating collagen synthesis, repair, and regeneration after tissue injury and may be secreted from monocytes, lymphocytes, and fibroblasts in the intestinal wall. In vitro studies have found that TGF-β1 increases collagen deposition by intestinal smooth muscle cells (Graham et al., 1990). Moreover, it has been reported that increased TGF-β1 levels in rat colonic tissues are associated with increased collagen deposition, whereas its neutralization with specific antibodies reduces collagen content in the colonic wall (Mourelle et al., 1998). Of course, TGF-β1 is unlikely to be the only fibrogenic cytokine, because it can stimulate macrophages and other cells to produce a variety of fibrogenic mediators, such as connective tissue growth factor. In addition, TGF-β1 is known to be controlled by a complex network of signaling cascades, including the upstream activator integrin and the downstream effector Smad (Leask and Abraham, 2004; Rieder and Fiocchi, 2009). Therefore, further work will be necessary to more fully elucidate the mechanism of CAI on colonic fibrosis.

In conclusion, the present study demonstrated that intragastric administration of CAI improves inflammation by reducing the production of proinflammatory cytokines, inhibiting NF-κB activation, and preventing colonic fibrosis in DSS-induced colitis. The findings suggest that CAI may be an alternate and effective strategy for treating human IBDS.

**Authorship Contributions**

*Participated in research design:* Guo, Ye, and Zhang.


*Contributed new reagents or analytic tools:* Li, Yu, and Zhu.

*Performed data analysis:* Guo and Hao.

*Wrote or contributed to the writing of the manuscript:* Guo, Ye, and Zhang.

**References**
