Immunopharmacology and Inflammation

Anti-inflammatory action of mollugin and its synthetic derivatives in HT-29 human colonic epithelial cells is mediated through inhibition of NF-κB activation

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Abstract

Mollugin is the active compound of Rubia cordifolia, which has been used as a traditional Chinese medicine for the treatment of various inflammatory diseases including arthritis and urotis. In the present study, we investigated for the first time the inhibitory effects and the mechanisms of action of mollugin (M1) and its synthetic derivatives (M2–M4) on tumor necrosis factor (TNF)-α-induced inflammatory responses in HT-29 human colon epithelial cells. Treatment with M1 and its derivatives M2–M4 significantly inhibited TNF-α-induced attachment of U937 mononuclear cells to HT-29 cells, which mimics the initial phase of colon inflammation. TNF-α-induced mRNA induction of the chemokines, monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8, and the intercellular cell adhesion molecule (ICAM)-1, which are involved in infiltration and migration of innate immune cells such as neutrophils and monocytes into tissue lesion sites, are also upregulated in inflammatory bowel disease, such as corticosteroids or mesalamine, have been shown to modulate the expression of ICAM-1 (Martinesi et al., 2008; Van Assche and Rutgeerts, 2005). In addition to chemokines, adhesion molecules such as intercellular adhesion molecule (ICAM)-1, which plays an important role in the recruitment of leukocytes at inflammation sites, are also upregulated in the intestinal mucosa of inflammatory bowel disease patients. Many therapeutically compounds used for the management of inflammatory bowel disease, such as corticosteroids or mesalamine, have been shown to modulate the expression of ICAM-1 (Martinesi et al., 2008; Van Assche and Rutgeerts, 2005).

Keywords: Mollugin, Chemokine, Interleukin adhesion molecule (ICAM)-1, NF-κB, Intestinal inflammation

1. Introduction

Inflammatory bowel disease is a chronic inflammatory condition of the gastrointestinal tract, resulting from impairment of the intestinal epithelial barrier function and a subsequent defect in adaptive immunity (Xavier and Podolsky, 2007; Tsianos and Katsanos, 2009). Infiltration and migration of innate immune cells such as neutrophils and monocytes into tissue lesion sites depends on the levels of cytokines and chemokines. In the pathogenesis of inflammatory bowel disease, accumulating evidence has revealed that altered expression of chemokines plays a critical role by activating and attracting leukocytes to the site of inflammation and upregulating adhesion molecules (Banks et al., 2003). Interleukin (IL)-8, an α-chemokine that attracts neutrophils, macrophages and T lymphocytes, is elevated in colon tissues of inflammatory bowel disease patients (Banks et al., 2003; Subramanian et al., 2008). Furthermore, the degree of colon inflammation correlates with the level of IL-8 expression (Mazzucchelli et al., 1994; Daig et al., 1996). Also, the level of monocyte chemoattractant protein (MCP)-1, a β2 chemokine that attracts monocytes and T lymphocytes to a lesion site, is increased in inflamed areas of inflammatory bowel disease patients (Grimm et al., 1996; Reinecker et al., 1995; Mazzucchelli et al., 1996). In addition to chemokines, adhesion molecules such as intercellular adhesion molecule (ICAM)-1, which plays an important role in the recruitment of leukocytes at inflammation sites, are also upregulated in the intestinal mucosa of inflammatory bowel disease patients. Many therapeutically compounds used for the management of inflammatory bowel disease, such as corticosteroids or mesalamine, have been shown to modulate the expression of ICAM-1 (Martinesi et al., 2008; Van Assche and Rutgeerts, 2005).

NF-κB is a key regulator of the inducible expression of many genes involved in immune and inflammatory responses in the gut (Dijkstra et al., 2002; Neurath et al., 1998). Activation of NF-κB has been observed in nuclear extracts from colonic biopsy samples in Crohn’s disease patients (Schreiber et al., 1998). In addition, drugs for inflammatory bowel disease therapy, such as mesalamine and glucocorticosteroids, also block the NF-κB signaling pathway (Egan et
et al., 1999; Auphan et al., 1995). Thus, more selective inhibition of NF-κB activation has been proposed as a treatment option for inflammatory bowel disease (Dijkstra et al., 2002).

Mollugin is a major bioactive component of Rubia cordifolia (Lu et al., 2007) which has been used as a traditional Chinese medicine for centuries. R. cordifolia is now listed in the Chinese Pharmacopoeia for the treatment of cough, inflammation of the joints, uterine hemorrhage, and uteritis. Pharmacological studies have demonstrated that the extracts of R. cordifolia have antibacterial, antioxidant, and anti-inflammatory activities (Basu et al., 2005; Cai et al., 2004; Tezuka et al., 2001). Even though R. cordifolia is used for inflammatory diseases, its exact mechanism of action has not been clearly demonstrated. In addition, the anti-inflammatory effects of purified mollugin itself have not been studied yet.

In the present study, we investigated for the first time anti-inflammatory effects and mechanism of action of mollugin (M1) and its synthetic derivatives (M2–M4) on intestinal inflammation by using an in vitro model of inflammatory bowel disease.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were from Hyclone-Pierce Co. (Hyclone, Logan, UT, USA), and Trypsin/EDTA was from Gibco Corp. (Paisley, Scotland, UK). 3-4-[5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and pyrrolidium dithiocarbamate (PDTC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mollugin and its synthetic derivatives, M1–M4, were synthesized as described previously (Fig. 1) (Lee et al., 2007).

2.2. Cell culture

HT-29 human colonic epithelial cells were grown in RPMI 1640 medium supplemented with 10% FBS and 100 µg/ml penicillin and 100 µg/ml of streptomycin in a humidified incubator under 5% CO2/95% air. The culture medium was replaced every other day. After reaching confluency, the cells were subcultured by trypsinization with trypsin–EDTA solution. U937 human pre- monocytic cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml of streptomycin.

2.3. Cell adhesion assay (monocytic cells and epithelial cells)

U937 monocytic cell adhesion to colonic epithelial cells was evaluated using human U937 pre-monocytic cells, which were prelabeled with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF/AM, 10 µg/ml) for 1 h at 37 °C. HT-29 cells cultured in 24-well plates were pretreated with M1–M4 or 5-aminosalicylic acid (5-ASA) for 1 h prior to incubation with TNF-α (10 ng/ml) for an additional 3 h. Cells were then co-incubated with BCECF/AM prelabeled U937 cells (1×104 cells/well) for 30 min at 37 °C. Non-adhering U937 cells were removed, and the HT-29 cells were washed twice with PBS. Cells were examined under an inverted fluorescence microscope equipped with a digital camera (TE2000-U, Nikon, Japan). For quantitative analysis, other sets of cells were lysed in 0.1% Triton X-100 in Tris (0.1 M), and the fluorescence was measured using a fluorescence-detecting microplate reader (Fluostar Optima, BMG Labtech GmbH, Germany) at excitation and emission wavelengths of 485 and 520 nm, respectively.

2.4. Measurement of cell viability

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) staining method. Briefly, cells at a density 1×104 cells/well were seeded in 96-well microtiter plates (Nunc, Denmark). After exposure to compounds, 20 µl MTT solution (5 mg/ml) was added to each well and the plates were incubated for additional 4 h at 37 °C. The medium containing the MTT solution was aspirated off and DMSO (200 µl) was added to solubilize the formazan salt formed. The amount of formazan salt was determined by measuring the optical density at 540 nm using a microplate reader (Versa MAX, Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was determined by the amount of MTT converted to formazan salt and expressed as a percent of the control culture.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Serum-starved HT-29 cells were pretreated with M1–M4 or 5-ASA for 1 h prior to incubation with 10 ng/ml TNF-α for an additional 3 h. Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and RT-PCR was performed as previously described (Beak et al., 2004). Briefly, cDNA was synthesized using a Ready-To-Go T-Primed First Strand kit (Amersham Biosciences, USA), and PCR was performed in the presence of 0.5U Taq DNA polymerase (Takara, Japan) using primers for human MCP-1, IL-8, ICAM-1 and GAPDH. PCR amplification conditions included denaturation at 94 °C for 4 min for the first cycle and for 1 min for the second cycle. The annealing and extension conditions used were: MCP-1 at 58 °C for 45 s and 35 amplification cycles of 72 °C for 60 s; IL-8 at 65 °C for 45 s and 19 amplification cycles of 72 °C for 90 s; and GAPDH 58 °C for 45 s and 30 amplification cycles of 72 °C for 45 s. Final extensions were performed at 72 °C for 10 min. The sequences of the primers used were as follows: human MCP-1 (sense 5′-ATG AAA GTC TCT GCC GCC CTT-3′, antisense 5′-TCA AGT CTT CGG AGT TTG GGT-3′); IL-8 (sense 5′-ATG TCT ACG GTG CTC GTG GCC GCC CTT-3′, antisense 5′-TCA AGT CTT CGG AGT TTG GGT-3′); IL-8 (sense 5′-ATG TCT ACG GTG CTC GTG GCC GCC CTT-3′, antisense 5′-TCA AGT CTT CGG AGT TTG GGT-3′); IL-8 (sense 5′-ATG TCT ACG GTG CTC GTG GCC GCC CTT-3′, antisense 5′-TCA AGT CTT CGG AGT TTG GGT-3′). PCR products were separated on 2% agarose gels containing ethidium bromide (0.5 µg/ml), and visualized and photographed using a gel documentation system (UVP, Cambridge,
The mRNA levels of all genes were normalized to that of GAPDH. The lengths of amplicons were 300, 289, 491, and 496 base pairs for MCP-1, IL-8, ICAM-1, and GAPDH, respectively.

2.6. NF-κB reporter gene dual-luciferase assay

HT-29 cells were transfected with 1 µg/ml of the NF-κB luciferase construct (firefly luciferase) in conjunction with 0.2 µg/ml of pRL-TK (renilla luciferase as a transfection control) using GeneJammer transfection reagent (Stratagene, CA, USA) according to the manufacturer's instructions. Cells were incubated with a transfection mixture at 37 °C for 3 h, mixed with the same volume of RPMI 1640 growth medium, and kept in an incubator at 37 °C overnight. These cells were then plated onto 24-well plates. After 24 h, the cells were pretreated with M1–M4 or 5-ASA and then incubated with 10 ng/ml TNF-α for 1 h. After 3 h, the cells were washed with PBS and then lysed by repeated freezing and thawing. Cells were then scraped gently and the lysates were centrifuged at 10,000 rpm for 5 min. Firefly and renilla luciferase activities were measured using a Dual-Luciferase Reporter Assay Kit (Promega Corporation, Madison, WI, USA) on a Turner TD20/20 luminometer (Turner Biosystems, CA, USA).

2.7. Statistical analysis

The data are expressed as mean ± S.E.M. of three independent experiments and analyzed using one-way analysis of variance (ANOVA) and the Student's t-test. P values of <0.05 were considered statistically significant.

3. Results

3.1. Inhibitory effects of M1 and its derivatives on TNF-α-induced adhesion of U937 monocyctic cells to HT-29 colonic epithelial cells

To investigate the effects of mollugin and its derivatives on intestinal inflammation, we examined the inhibitory effects of mollugin and its derivatives on TNF-α-induced adhesion of U937 pre-monocytic cells to HT-29 human colonic epithelial cells, which mimics the initial step of colon inflammation. We first performed the experiment with M1, and selected a 20 µM concentration from the dose–response curve of M1, of which the effect was better than 5-ASA (20 mM), a drug that is commonly used for the treatment of inflammatory bowel disease (Fig. 2A). As shown in Fig. 2B and C, mollugin (M1) and its derivatives, M2, M3, and M4, significantly inhibited TNF-α-induced monocyte–epithelial adhesion. M1 (20 µM) was the most potent among the derivatives tested. The compounds M1–M4 at up to 20 µM concentration did not affect the cell viability (Fig. 2D).

3.2. M1 and its derivatives suppress TNF-α-induced expression of the chemokines, IL-8 and MCP-1, and the adhesion molecule, ICAM-1, in a NF-κB-dependent manner

We next examined which inflammatory molecules involved in the initial process of inflammation were altered by the mollugin compounds. Treatment of HT-29 colonic epithelial cells with TNF-α significantly increased the mRNA expression of MCP-1 (Fig. 3A and B) and IL-8 (Fig. 3A and C), chemokines that play important roles in the activation and recruitment of leukocytes during the inflammatory process (Yamamoto-Furuhasho, 2007). M1–M4 significantly suppressed the TNF-α-induced expression of MCP-1 and IL-8, with M1 being the most effective and M3 the least effective. The effects of M1–M4 on TNF-α-induced expression of ICAM-1 were similar to their effects on chemokine expression (Fig. 3A and D). M1 showed the strongest effect, which was comparable to that of 5-ASA, whereas M3 showed a weak effect.

Since the expression of inflammatory cytokines is closely associated with activation of NF-κB (Banks et al., 2003), we also examined whether the inhibitory effects of mollugin and its derivatives on the pro-inflammatory cytokines were due to their ability to suppress TNF-α-induced NF-κB activation. As shown in Fig. 4A, TNF-α-induced NF-κB transcriptional activity in HT29 cells was significantly suppressed by M1–M4. The order of activity was M1 > M2 > M4 > M3. In addition, the inhibitory effect of M1 (1 µM) on the TNF-α-induced NF-κB activation was similar to that of PDTC (50 µM), a NF-κB inhibitor. Treatment of HT-29 cells with M1 (1 µM) in the presence of PDTC (50 µM) synergistically suppressed both TNF-α-induced NF-κB activation (Fig. 4B) and U937 monocyctic cell adhesion to HT-29 cells (Fig. 4C).

4. Discussion

Excessive infiltration of immune cells to tissue lesion sites initiates and aggravates inflammation. Thus, chemokines, which activate and recruit leukocytes to an injured site, are target molecules for new drug development in the treatment of chronic inflammatory diseases such as rheumatoid arthritis (Amat et al., 2006; Haringman et al., 2003). Altered production and signaling of chemokines and cytokines have been implicated in the pathogenesis of inflammatory bowel disease. Similar to other chronic inflammatory diseases, excessive cytokine activity in inflammatory bowel disease may serve as a new drug target. In the effort to develop inflammatory bowel disease drugs, infliximab, an anti-TNF-α antibody, has successfully been launched as a potent inflammatory bowel disease drug. In addition to TNF-α, chemokines implicated in inflammatory bowel disease have become drug targets for new approaches in the treatment strategy for inflammatory bowel disease patients (Zhong et al., 2008). In the present study, we searched for chemokine-targeting drug candidates for the treatment of inflammatory bowel disease using M1–M4, i.e., mollugin and its synthetic derivatives. Among the compounds, M1 was the most potent and efficacious drug candidate for suppressing TNF-α-induced expression of the chemokines, MCP-1 and IL-8, and the adhesion molecule ICAM-1, as well as monocyte adhesion to colonic epithelial cells.

The regulatory sites in chemokine and adhesion molecule genes have NF-κB binding sites. NF-κB activation is strongly induced in the inflamed gut of patients with inflammatory bowel disease, and the activated NF-κB level correlates significantly with the severity of intestinal inflammation (Jobin and Sartor, 2000). Furthermore, antioxidants and agents that inhibit the NF-κB pathway have been considered proper therapeutic drugs in the treatment of inflammatory bowel disease (Neurath and Meyer zum Büschenfelde, 1996). In contrast to the constitutively expressed chemokines which direct baseline leukocyte trafficking, the inducible chemokines such as IL-8 profoundly attract leukocytes to local sites and aggravate inflammation (Laing and Seconombe, 2004). Previously, we reported that a drug suppressing IL-8 expression effectively ameliorated intestinal inflammation in a rat model of inflammatory bowel disease, and the action of the drug was...
mediated through the NF-κB pathway (Thapa et al., 2008). Likewise, in the present study, the NF-κB inhibitory activities of M1–M4 correlated with their ability to suppress the chemokine expression in HT-29 cells and the inflammatory process in vitro. In addition, the synergistic effects of M1 and PDTC on TNF-α-induced NF-κB activation in HT-29 cells as well as adhesion of U937 monocytic cells to HT-29 epithelial cells indicate that M1 acts as an NF-κB inhibitor. Furthermore, M1 was much more efficacious than 5-ASA, a conventional drug for inflammatory bowel disease. Inhibition of NF-κB activation has been increasingly proposed for the mechanism of 5-ASA action (Bantel et al., 2000; Kim et al., 2006; Yan and Polk, 1999) in addition to its inhibitory action in 5-lipoxygenase and cyclooxygenase (Nikolaus et al., 2000).

Taken together, these results indicate that as an NF-κB inhibitor, M1 may be a prominent new drug candidate in the treatment strategy for inflammatory bowel disease.

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References


Jobin, C., Sartor, R.B., 2000. NF-kappa B inhibitory activities of M1 may be a prominent new drug candidate in the treatment strategy for inflammatory bowel disease.

Fig. 3. Inhibitory effects of M1 and its synthetic derivatives (M2–M4) on TNF-α-induced pro-inflammatory cytokine expression. HT-29 cells were pretreated with 20 µM M1–M4 for 1 h, followed by 10 ng/ml of TNF-α for 3 h. The mRNA expressions of inflammatory mediators were measured by RT-PCR. The PCR products were separated in 2% agarose gel (A). The bands of PCR products specific to MCP-1 (B), IL-8 (C), and ICAM-1 (D) were quantitated by densitometry, and expressed as relative value to GAPDH.
Fig. 4. Inhibitory effects of M1 and its synthetic derivatives (M2–M4) on TNF-α-induced NF-κ-B transcriptional activity and monocyte adhesion to epithelial cells. (A and B) HT-29 cells transfected with NF-κB plasmid were treated with M1–M4 or in the presence of TNF-α. The NF-κB gene transcription activity was measured with a luciferase assay kit (Dual-Luciferase Activity assay system; Promega). (C) HT-29 cells were co-treated with M1 and PDTC in the presence of TNF-α. The adhesion of BCECF fluorescence-labeled U937 cells to HT-29 colon epithelial cells was detected as described in Fig. 1. Data represent means ± S.E.M. from three independent experiments.

*P<0.05, compared to untreated control. **P<0.05, compared to TNF-α-treated group.


