RESEARCH ARTICLE

Demethoxycurcumin from *Curcuma longa* Rhizome Suppresses iNOS Induction in an *in vitro* Inflamed Human Intestinal Mucosa Model

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Abstract

Background: It is known that inducible nitric oxide synthase (iNOS)/nitric oxide (NO) plays an integral role during intestinal inflammation, an important factor for colon cancer development. Natural compounds from *Curcuma longa* L. (Zingiberaceae) have long been a potential source of bioactive materials with various beneficial biological functions. Among them, a major active curcuminoid, demethoxycurcumin (DMC) has been shown to possess anti-inflammatory properties in lipopolysaccharide (LPS)-activated macrophages or microglia cells. However, the role of DMC on iNOS expression and NO production in an *in vitro* inflamed human intestinal mucosa model has not yet been elucidated. This study concerned inhibitory effects on iNOS expression and NO production of DMC in inflamed human intestinal Caco-2 cells. An *in vitro* model was generated and inhibitory effects on NO production of DMC at 65 μM for 24-96 h were assessed by monitoring nitrite levels. Expression of iNOS mRNA and protein was also investigated. DMC significantly decreased NO secretion by 35-41% in our inflamed cell model. Decrease in NO production by DMC was concomitant with down-regulation of iNOS at mRNA and protein levels compared to proinflammatory cytokine cocktail and LPS-treated controls. Mechanism of action of DMC may be partly due to its potent inhibition of the iNOS pathway. Our findings suggest that DMC may have potential as a therapeutic agent against inflammation-related diseases, especially in the gut.

Keywords: Curcuminoids - inflamed human intestinal Caco-2 cells - iNOS/NO - *in vitro* model

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Introduction

Intestinal mucosa acts as a barrier to protect the gut from various chemicals and foreign pathogens. Inflammation of the gut is a protective process to maintain gut integrity and functions, while dysregulation of this process can lead to severe intestinal disorders. Intestinal inflammation can occur in disease states such as inflammatory bowel disease (IBD), i.e. ulcerative colitis and Crohn's disease. IBD has been taken into account as one cause of colon cancer (Grivennikov, 2013). When inflammation occurs, it also produces several inflammatory mediators and these mediators are components of inflammatory microenvironment that involve cancer formation and progression as well as the response to therapy (Coussens and Werb, 2002). Previous studies have been suggested that NO contributes to the development of carcinogenesis by eliciting chronic inflammation conditions (Jaiswal et al., 2001). NO is one of the inflammatory mediators. It is also a free radical produced from immune cells and other cell types such as epithelial cells in order to get rid of pathogens (Eckmann et al., 2000). Enhanced expression of the inducible nitric oxide synthase (iNOS) with the sustained overproduction of the free radical nitric oxide (NO) is one of the most consistent and dramatic findings in both experimental and human IBD (Grisham et al., 2002). In the immunologic IBD mouse model, iNOS, 8-nitroguanine, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and proliferating cell nuclear antigen (PCNA) were expressed in epithelial cells indicating nitrative and oxidative DNA damage followed by proliferation of these cells, which may contribute to IBD carcinogenesis (Ding et al., 2005). N-acetylcysteine inhibited chronic ulcerative colitis-associated colorectal adenocarcinoma development, possibly by reduction of nitrative DNA damage produced via iNOS in a murine model (Seril et al., 2002). Moreover, the expression of iNOS mRNA was significantly increased during neoplastic transformation of colonic mucosa in ulcerative colitis patients (Svec, et al., 2010). These data implicate that iNOS/NO plays a role

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in inflammation-associated carcinogenesis of the gut.

Demethoxycurcumin (DMC) is one of the main active constituents of *Curcuma longa* L. (Zingiberaceae). Anti-inflammation effect of DMC has been demonstrated in different models both *in vitro* and *in vivo* studies. DMC showed higher potency in suppressing inflammation in lipopolysaccharide (LPS)-induced NO production, iNOS, cycloxygenase-2 (COX-2) and nuclear factor-κB (NF-κB) activity in a RAW 264.7 macrophage cell line than that of bisdemethoxycurcumin (BDMC) and *in vivo* carrageenan-induced paw edema in mice (Guo et al., 2008). Concomitantly, DMC exhibited the strongest inhibitory activity on NO and TNF-α production in LPS-activated microglia compared to curcumin (CUR) and BDMC (Zhang et al., 2008). Preclinical and clinical research has shown mainly CUR and its anti-inflammatory properties. CUR may have potential as a therapeutic agent in diseases such as inflammatory bowel disease, pancreatitis, arthritis, and chronic anterior uveitis, as well as certain types of cancer (Jurenka, 2009) but little is known about the natural demethoxy analog of CUR, DMC, has the similar effects as CUR especially in the gut.

In order to generate inflammatory microenvironment in an *in vitro* model of intestinal inflammation, differentiated human intestinal epithelial cell line Caco-2 has to be exposed to three major cytokines, IL-1β, TNF-α, IFN-γ in the presence of LPS (Van De Walle et al., 2010). As DMC has not yet been investigated for its role in intestinal inflammatory model, the purpose of this study is to investigate the effect of DMC on nitrite secretion and iNOS expression in an inflamed human intestinal mucosa model.

Materials and Methods

Culture of differentiated human colon cancer cells (Caco-2 cells) and induction of NO secretion

To establish *in vitro* model of the human intestinal barrier, Caco-2 cells (Cell Line Service, Germany) at passage number 52 were seeded at a density of 4×10^4 cells/cm^2 in a complete media containing DMEM/Ham's F12 (PAA, Austria) supplemented with 10% fetal bovine serum (Biochrome AG, Germany), 1% penicillin/streptomycin (PAA, Austria) and 2 mM stable glutamine (PAA, Austria). Cells were grown in humidified 5% CO_2 atmosphere at 37°C and cell culture medium was changed every second day until the cells were fully differentiated (day 21) (Van De Walle et al., 2010). To induce NO secretion in differentiated Caco-2 cells, these cells were treated with the inflammatory mediators 50 ng/ml IL-1β, 25 ng/ml TNF-α, 50 ng/ml IFN-γ, and 0.5 μg/ml LPS for 24, 48, 72, and 96 h. Culture media was subjected for Griess assay for nitrite levels.

Griess assay

Culture media was centrifuged at 3,000 rpm, 4°C for 10 min and the supernatant was subjected for nitrite measurement by Griess assay. Briefly, 150 μl of collection medium was mixed with 130 μl deionized water and 20 μl Griess reagent (Bezerra et al., 2004). After 30 min, the optical density was measured at 548 nm. Nitrite level was determined in μM from a nitrite standard curve with linear relation of $r^2>0.990$.

Cytotoxicity test

DMC was obtained from *C longa* rhizomes by the method described (Changtam et al., 2010). Differentiated Caco-2 cells cultured in 96-well plate were treated with DMC at various concentrations. DMSO treated cells were used as vehicle control. All treated cells were incubated for 24 h at 37°C in humidified 5% CO_2 atmosphere. Cell viability was performed by MTT assay. Data were expressed from three independent experiments in triplicate.

*DMC treatment in an **in vitro** model of inflamed human intestinal mucosa*

Caco-2 cells were seeded at a density of 4×10^4 cells/cm^2 and incubated for 21 days to obtain differentiated Caco-2 cells as previously described (Van De Walle et al., 2010). Differentiated cells were treated with DMC at a concentration of 65 μM for 4 h followed by incubating the cells with the inflammatory mediators (50 ng/ml IL-1β, 25 ng/ml TNF-α, 50 ng/ml IFN-γ, and 0.5 μg/ml LPS) for 24, 48, 72, and 96 h. Culture supernatant was subjected for nitrite measurement by Griess assay as previously described. Cells were harvested and evaluated for iNOS mRNA and protein expression by Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis, respectively.

RT-PCR analysis of iNOS mRNA

The total RNA was extracted by using RNeasy Mini kit (Qiagen, Germany) according to the user's manual. The concentration of total RNA was measured by NanoDrop spectrophotometer (NanoDrop™ 1000, USA). Then 5 μg total RNA was converted to cDNA by Omniscript RT Kit (Qiagen, Germany). Total cDNA of 500 ng/ml was applied to Thermal cycler PCR machine to amplify DNA template. The sequences of the PCR primers were as follows: 5'-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA-3'(forward) and 5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG-3'(reverse) for iNOS (Chunglok et al., 2004) and 5-TGG ATA TTG TTG CCA TCA ATG ACC-3'(forward) and 5'-GAT GGC ATG GAC TGT GGT CAT G-3'(reverse) for GAPDH (Chunglok et al., 2004). Finally, PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized under ultraviolet light.

Western blot analysis of iNOS protein expression

Cells were harvested and washed three times with ice-cold phosphate buffered saline (PBS). For positive control, RAW 264.7 macrophages (kindly supplied by Dr. Poonsit Hiransai, School of Allied Health Sciences and Public Health, Walailak University) were treated with 2 μg/ml LPS for 24 h. Cells were lysed by sonication in sonicacation buffer containing protease inhibitors (150 mm Tris-HCl, pH 7.8, 10 mM PMSF, 0.2 mM Na_3VO_4, 0.01 M NaF, 0.014 mM chymostatin, 99.3 KU/ml aprotinin, and 0.01 mg/ml pepstatin A). Cell lysate was further centrifuged at 10,000 rpm at 4°C for 15 min. Supernatants

was collected and protein concentrations were measured by using Bradford reagent kit (BioRad, USA). iNOS protein levels in cell lysates were then determined by immunoblot analysis. Briefly, 80 μg protein of each sample was loaded to 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) filters. The membranes were blocked with blocking buffer, 5% milk in Tris-buffered saline and Tween 20 (TBST) for 1h at room temperature and then were incubated with anti-iNOS antibody (1:500) (Cell Signaling Technology, USA) in 5% BSA, overnight at 4°C. After washed three times with washing buffer (TBST), the washed membranes were incubated with anti-mouse secondary antibody (1:5000) (Cell Signaling Technology, USA) for 1h at room temperature. Chemiluminescent system using Hyperfilm (Amersham, UK) and ECL plus (Amersham, UK) detected the specific bands of proteins. For the internal control, membranes were stripped and reprobed with anti β-actin antibody (1:5000) for 1h.

Statistical analysis

Statistical analysis was analyzed by using One-Way ANOVA, independent t-test and Mann-Whitney U-test. Differences were considered statistically significant when the probability value $p<0.05$.

Results and Discussion

Although a large body of literatures focus on anti-inflammatory properties of CUR especially in the gut, little is known about DMC. *in vitro* model of the human intestinal inflammation was generated in order to study the role of DMC in the gut. In the present study, DMC, one of the curcuminoids, (Figure 1) at concentrations of 16.25-65.00 μM did not affect cell viability of differentiated Caco-2 cells (Figure 2). Cytotoxic effect of DMC was observed at concentrations of 130 μM and 150 μM. This may be a potential anti-proliferative effect against colon cancer Caco-2 cells of DMC. The maximal concentration of DMC (65 μM) that do not affect cell viability was chosen to treat the cells in the following experiment.

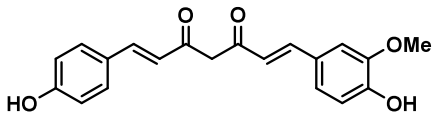

Figure 1. The Structure of Demethoxycurcumin (DMC)

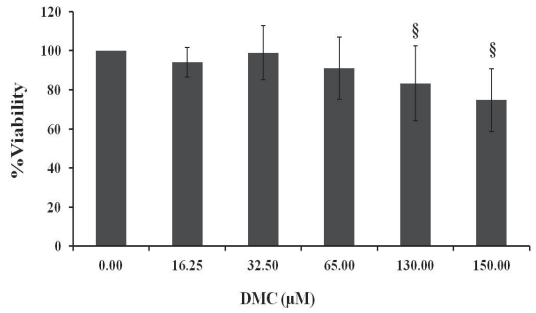

Figure 2. Cell Viability of Differentiated Caco-2 Cells upon DMC Treatment. Values were expressed as Mean±SD in three-independent experiments in triplicate. Data were analyzed using ANOVA. Difference with control (untreated cells) is marked with §when $p<0.05$

DMC at 65 μM significantly reduced NO secretion to approximately 35%, 41%, 38% and 37% compared to the inflamed Caco-2 cells at 24, 48, 72, and 96 h, respectively (Figure 3). Direct effect of DMC may contribute to the suppression of iNOS at mRNA and consequently iNOS protein expression. Further investigation is on the expression of iNOS at both transcriptional and translational levels. The results showed that DMC markedly down-regulated the expression of iNOS at mRNA (Figure 4) and protein levels (Figure 5). The expression of iNOS at mRNA levels was markedly decreased up to 40% compared to the inflamed condition. This was concomitant with the reduction of iNOS protein to approximately 57%.

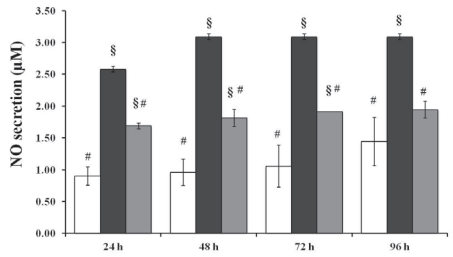

Figure 3. NO Inhibitory Effect of DMC. Caco-2 cells were pretreated with DMC for 4 h followed by 50 ng/ml IL-1β, 25 ng/ml TNF-α, 50 ng/ml IFN-γ, and 0.5 μg/ml LPS for 24, 48, 72, and 96 h. **N**itrite level was measured by griess assay. Data were analyzed using independent T-Test. Difference with control (Untreated Cells) and inflamed condition are marked with § or # when $p<0.05$

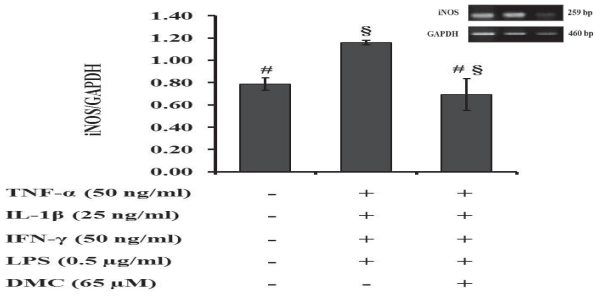

Figure 4. Effect of DMC on iNOS mRNA Expression. Differentiated Caco-2 cells were pretreated with DMC for 4 h followed by inflammatory mediators for 96 h. RT-PCR was performed for iNOS mRNA and GAPDH was used as internal control. Difference with control (untreated cells) and inflamed condition is marked with § or # when $p<0.05$

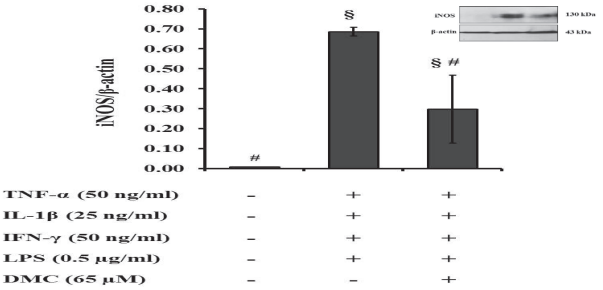

Figure 5. Effect of DMC on iNOS Protein Expression. Differentiated Caco-2 Cells were pretreated with DMC for 4 h followed by inflammatory mediators for 96 h. Western blot analysis for iNOS protein expression was performed. B-actin was used as internal control. Difference with control (untreated cells) and inflamed condition is marked with § or # when $p<0.05$

DMC strongly regulated NO production in an inflamed intestinal cell model and diminished iNOS expression at both transcriptional and translational levels.

Our present study reports that DMC can modulate *in vitro* model of intestinal inflammation by down-regulation of iNOS induction at both the transcriptional and translational levels, leading to a decrease in NO production. As DMC can down-regulate iNOS/NO, our results are consistent with the previous reports on the inhibition of iNOS at mRNA and protein levels by DMC in LPS-activated rat primary microglial cells (Zhang et al., 2008) and RAW 264.7 macrophage cells (Guo et al., 2008; Kaewkroek et al., 2010). DMC may have the direct effect in inhibition of NO production via iNOS pathway as NO-scavenging ability of DMC is very weak (Zhang et al., 2008). The mechanism of action of DMC may be partly due to its potent down-regulation of intracellular ROS level and thus leading to inactivation of NF-κB and MAPKs as well as inhibiting the expression of iNOS, TNF-α, and IL-1β (Zhang et al., 2010). These results are consistent with the anti-inflammatory property of DMC in inhibiting iNOS and COX-2 expression by the inhibition of NF-κB activity (Guo et al., 2008). It has been shown that all of the three curcuminoid pigments (CUR, DMC, and BDMC) significantly suppressed nitric oxide (NO) production in LPS-activated microglia and the relative potency was DMC>BDMC>CUR (Zhang et al., 2008). The absence of one methoxy group may enhance the inhibitory effect of NO production. The relative position of 4-phenolic hydroxy group and 3-methoxy group may be very important for the inhibitory effect of DMC (Guo et al., 2008; Zhang et al., 2008). Finally, concentrations of the inflammatory stimuli and DMC used to treat Caco-2 cells were found not to be cytotoxic. Our results support the potential use of DMC for treating intestinal inflammation.

This is the first report of the potent inhibitory activity of DMC in inhibiting NO secretion through the suppression of iNOS induction in an *in vitro* inflamed human intestinal mucosa model. This may be one plausible mechanism of DMC on its anti-inflammatory activity. DMC may have therapeutic potential for the treatment of IBD accompanied by inflammation of the gut, which in turn the risk of colon cancer. Further investigation is needed to determine the molecular mechanisms and pathways related to its inhibitory effects of DMC.

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