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Curcumin Suppresses the Activity of Inhibitor-κB Kinase in an *in vitro Inflamed Human Intestinal Mucosa Model* by *S*-nitrosylation

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Abstract In previous study, we found curcumin to possess anti-inflammatory properties in lipopolysaccharide (LPS)-induced macrophage cells due to the involvement of curcumin and *S*-nitrosylation in the NF-κB pathway. However, the role of curcumin on regulation of NF-κB signaling pathway through *S*-nitrosylation in an *in vitro inflamed human intestinal mucosa model* has not yet been elucidated. This study aimed to concern inhibitory effects of curcumin on NF-κB pathway in two type of inflamed human intestinal cells, Caco-2 and HT-29. Western blot presented the protein expression of iNOS can be reduced by treated curcumin with 30 μM for 12h. Consistently, pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα and IFN-γ was also repressed. The results also shows curcumin reduced the amount of nitrite and nitrate in inflamed human intestinal cells, Caco-2 and HT-29, maintained total *S*-nitrosylation level on proteins. Furthermore, the protection of *S*-nitrosylation on IKKβ in inflamed Caco-2 and HT-29 cells by curcumin caused the repression of IκB phosphorylation and NF-κB activation. In conclusion, this study verified that curcumin-mediated *S*-nitrosylation may be as an important regulator for anti-inflammation in an *in vitro inflamed human intestinal mucosa model*.

Keywords: *curcumin, S-nitrosylation, inflamed human intestinal cells, NF-κB, IκB, IKK, nitric oxide*

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1. Introduction

Nitric oxide (NO), a short-lived free radical generated by nitric oxide synthase (NOS), is a pleiotropic secondary messenger to participate in diverse biological processes such as inflammation and tumor progression [1]. Local inflammation induced by NO triggers diverse abnormal characteristics in cancer cells [2,3]. Persistent activation of inducible nitric oxide synthase (iNOS) resulted from chronic inflammation promotes transformation of the adenoma to early stage of carcinoma [4]. Meanwhile, the physiological activation of iNOS also facilitates *S*-nitrosylation and downstream enzymatic activation [5].

For inflammation, *S*-nitrosylation occurs in the most proteins and causes it to lose functions, including inhibitor-κB kinase (IKK), iNOS, NF-κB, matrix metalloproteinases (MMPs) and cysteine-aspartic proteases (caspases) [6,7,8]. The results of numerous studies show that the NO suppresses IKK through *S*-nitrosylation, which prevents the transcription of NF-κB [9]. In the complex regulation of NF-κB, the inhibitory molecule I-κB is a key step [10].

Curcumin, a natural polyphenolic compound from the Curcuma longa plant, has been studied to possess diverse pharmacological properties, such as antioxidant, anti-inflammatory, and anti-cancer activities [11,12,13,14]. The antioxidant and anti-inflammatory properties of curcumin have been described to decrease production of proinflammatory cytokines, such as TNFα, IL-1, and IL-6 [15], reduce mRNA and protein level of iNOS [16], and sequestrate free radicals [17]. In addition, curcumin also functions as a direct scavenger of NO [18,19], suppresses NO-derived nitrite formation [20], and inhibits protein activity [13].

In the previous study, we provided the data that reveals curcumin-mediated *S*-nitrosylation may be as an important regulator for anti-inflammation in LPS-induced macrophage cells [21,22]. However, the role of curcumin on regulation of NF-κB signaling pathway through *S*-nitrosylation in an *in vitro inflamed human intestinal mucosa model* has not yet been elucidated. In order to confirm that curcumin represses the activity of inhibitor-κB kinase protein *S*-nitrosylation more accurately, an *in vitro inflamed human intestinal mucosa model* was used to verify curcumin maintains the IKK *S*-nitrosylation here. In this report, we investigated whether curcumin affects *S*-nitrosylation of IKK and subsequent NF-κB pathway activation by using two type of inflamed human intestinal cells, Caco-2 and HT-29.

2. Materials and Methods

2.1. Reagents

Roswell Park Memorial Institute (RPMI) 1640 Medium, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen (Grand Island, NY, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). The Total Nitric oxide (NO) and Nitrate/Nitrite Assay Kit, antigens, and goat anti-mouse IgM Cy5-conjugated antibody for the cytokine microarray chips were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The microarray chips were obtained from CEL & Associates, Inc. (Los Angeles, CA, USA). The antibodies, including NF-κB, IκB, IKK, iNOS, and β-Actin, were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). The Bicinchoninic Acid (BCA) Protein Assay Kit, Bradford Protein Assay Kit, and PEO-iodoacetyl-biotin were obtained from Pierce (Rockford, IL, USA). Other chemicals were purchased from Sigma -Aldrich (St. Louis, MO, USA).

2.2. Cell Culture

Human intestinal cells (Caco-2 and HT-29) were obtained from the ATCC (Manassas, VA, USA) and grown in an uncoated plastic cell culture dish (150 × 25 mm) with 20 ml of RPMI 1640 or DMEM medium containing 10% bovine fatal serum (FBS) and 1% antibiotics (Invitrogen, Grand Island, NY, USA). The medium was changed every 2 d until the cells were fully differentiated (day 21) [23]. All cell cultures were conducted at 37 ℃ in a 5% CO_2 incubator. At confluence, cells were passaged using trypsinization.

2.3. Determination of Cytotoxicity with Curcumin on Cell Viability

To determine the effects of curcumin on Caco-2 and HT-29 cells growth, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT assay). Cells were seeded in 96-well cell culture plates with approximately 1×10^4 cells per well. After overnight culture, the cells were treated with various concentrations of curcumin that is 0, 1, 5, 10, 15, 30, 60, 120 and 240 μM (containing 0.4% of DMSO to resolve curcumin) for 24 and 48 h. The cells were subsequently incubated with 5 mg/ml of MTT working solution for 4 h at 37 ℃, followed by treatment with 20 μl of DMSO to dissolve the crystals [24]. The number of independent experiments (n=3) for cytotoxicity of curcumin on cell viability detection was determined based on statistical power calculation [25].

2.4. Determination of Total *S*-nitrosylated Proteins

To detect the variation of total *S*-nitrosylated protein expression with 30 μM curcumin on Caco-2 and HT-29 cells that was treated with LPS and curcumin. The 1×10^6 cells were seeded overnight in a 150 × 25 mm culture dish with 20 ml of RPMI1640 or DMEM medium. After the cells were attached to the dish completely, discarded the cultured medium and washed gently with 1X phosphate buffered saline (PBS) for 3 times. The cells were cultured with a fresh medium that contained LPS with 0.1μg/ml. The curcumin (Sigma-Aldrich, St. Louis, MO, USA) that was diluted from the stock was subsequently added and the final concentrations of the medium was 30 μM. At the end of 12h culture periods, the culture medium was collected and centrifuged at 12 000 rpm for 5 min for the preparation of cytokine microarray, nitrite and nitrate production assay. The total protein from Caco-2 and HT-29 cells were collected individually by treated the lysis buffer containing the HEN buffer (250 mM HEPES at pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine), 1% NP-40, 150 mM NaCl, 1 mM PMSF, and 1/100 (v/v) protease inhibitor cocktail set III. The protein concentration was determined using the BCA Protein Assay Kit. All procedures of the detection on *S*-nitrosylated proteins were performed in accordance with Chen et al [26]. *S*-nitrosylated proteins were labeled with PEO-iodoacetyl-biotin using the biotin switch method (BSM). 2 μg of biotinylated protein was separated by 10% SDS PAGE and transferred to a polyvinylidene fluoride membrane. After blocked and treated with the anti-biotin monoclonal antibody (1:5000) for 1h at room temperature. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system.

2.5. Analysis of Expression of *S*-nitrosylated IKK Using Immunoprecipitation

To determine the variation of *S*-nitrosylated IKK treated with 30 μM of curcumin on LPS-induced human intestinal cells, Caco-2 and HT-29 were treated with or without curcumin for 12h. A total of 250 μg of *S*-nitrosylated proteins were obtained from Step 2.4., and resuspended with an avidin loading buffer (2X PBS, 40 mM NaH_2PO_4 at pH 7.2 and 300 mM NaCl). Protein concentration was adjusted to 2 mg/ml. These *S*-nitrosylated proteins were purified with streptavidin agarose (Sigma-Aldrich) for incubation for 1h. After washing with an avidin wash buffer I (1X PBS at pH7.2), wash buffer II (50mM ABC at pH8.3 and 20% methanol) and 1 ml Milli Q water were used. All samples were eluted by an avidin eluting buffer (30% ACN, 0.4% TFA). The purified sample was separated by 10% SDS PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked and incubated with an IKK primary antibody. The condition was set according to the instructions provided by the manufacturer. The protein bands were visualized using an enhanced chemiluminescence detection system.

2.6. Detection of the Effect of Curcumin on NF-κB Pathway Using Western Blotting.

To profile the relationship of *S*-nitrosylated IKK and curcumin on LPS-induced human intestinal cells, the proteins of the NF-κB pathway, which included iNOS, IKK, NF-κB, IκB, and phosphorylated inhibitor-κB

(p-IκB), were detected using western blotting. The total lysates were prepared in Step 2.4.; however, this step was not used for BSM. Equal amounts of protein from each lysate were separated by 10% SDS PAGE and transferred to a nitrocellulose membrane, blocked, and subsequently incubated with the relevant blotting antibodies.

2.7. Determination of Inflammatory Cytokines Expression

The cytokine microarray including interleukin-1β (IL-1), interleukin-6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were manufactured by the robotic Cartesian/Microsys arrayer (Cartesian technologies, Irvine, CA, USA). The procedure of the microarray chips was performed in accordance with Quintana et al [27]. The chips were stored at 4℃ overnight, and blocked for 1 h at 37℃ with 1% BSA. After blocking, each chip was washed with 1X PBS and 1X PBST (1X PBS contained 0.025% tween-20) for 3 min and incubated for 1 h at room temperature with 10 μl of the test cultured medium. The arrays were subsequently washed and incubated for 1 h with a 1:500 dilution of a goat anti-mouse IgM Cy5-conjucated antibody. After washed and dried, Arrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The rules were recorded as TIFF files.

2.8. Nitrite and Nitrate Production Measurement

Nitrite and nitrate production in treated LPS human intestinal cells was determined using a commercial nitrite/nitrate colorimetric assay kit (Cayman chemical company, Ann Arbor, MI). All procedures were conducted according to the instructions of the manufacturer. The cell culture medium was mixed with a Griess reagent and incubated for 10 min at 37℃. The absorbance was measured at 540 nm with a 96-well plate reader. The nitrite and the nitrate concentration were calculated using sodium nitrite/nitrate as a standard.

2.9. Statistical Analysis

The data were reported as mean ± SEM, using one-way analysis of variance and Student's Newman-Keuls test for post hoc comparisons to determine differences between the control and experimental groups. A *p* value of less than 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of Various Levels of Curcumin on Human Intestinal Cells Viability

The death of Caco-2 and HT-29 cells were induced with curcumin in a dose and time-dependent manner (Figure 1). Concentrations of 30 μM were selected to present the effects of curcumin on LPS-treated Caco-2 and HT-29 cells.

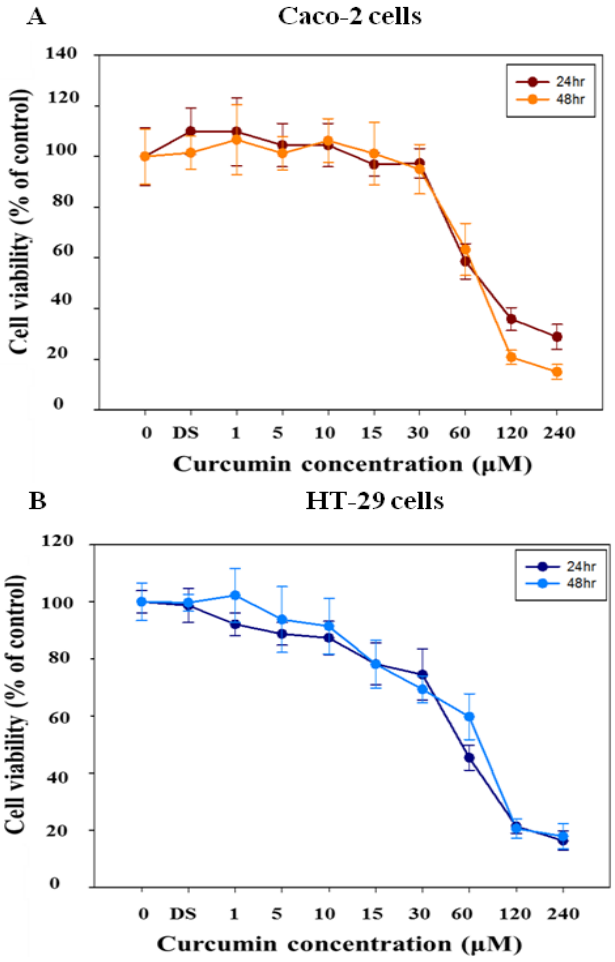

Figure 1. The cytotoxicity of curcumin on Caco-2 and HT-29 cells. Both Caoco-2 (A) and HT-29 cells (B) were incubated with samples for 12 h or 48 h and viability was assessed using an MTT assay. Experiments were repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3)

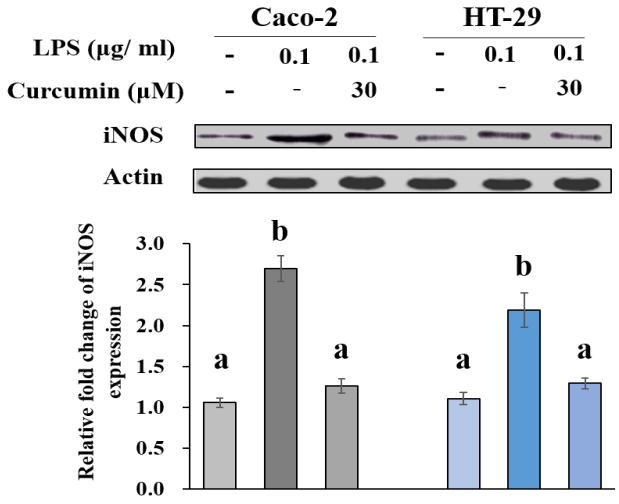

Figure 2. The repressionof iNOS in LPS-induced human intestinal cells by curcumin. Total lysates was extracted from LPS-induced Caco-2 and HT-29 cellsof each experimental group at 12hindividually. The expression of iNOS was detected by Western blot, and β-actin was as the loading control. Experiments were repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p < 0.05$) at corresponding concentrations between different treatments

3.2. Curcumin Inhibits LPS-induced Inflammatory Factors and NO-derived Nitrite Production in Human Intestinal Cells

Caco-2 and HT-29 cells were treated with LPS (0.1 μg/ml), and 30 μM of curcumin was subsequently added for 12h. The cell lysates was subjected to Western blot assay using an iNOS antibody. Results showed curcumin prevents LPS-induced iNOS expression (Figure 2). To verify these results, nitrite (NO_2) and nitrate (NO_3), the NO oxide production was examined from cell cultured medium. The data reveals curcumin significantly diminished NO oxide production (Figure 3A-B).

Numerous studies have addressed abnormal cytokine expression, especially the inflammation. In previous decades, curcumin is thought to have multiple benefits to modulate inflammation [28,29]. To confirm the ability of curcumin to inhibit inflammation, we investigated the correlation between curcumin and cytokine expression. The cultured medium was collected from LPS- induced Caco-2 and HT-29 cells, and 30 μM curcumin were added simultaneously at the end of 12h culture periods. After the supernatant was centrifuged at 15 000 rpm for 1 min at 4 ℃, it was incubated with the microarray chip for 1 h at room temperature. Cytokine expression was detected by the scanner after combining with the IgM Cy5-conjucated antibody. The results showed that curcumin attenuated the inflammation by decreasing pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ at 12 h (Figure 4A-B). The results as well as previous reports that indicates curcumin can decrease pro-inflammatory cytokine [30,31].

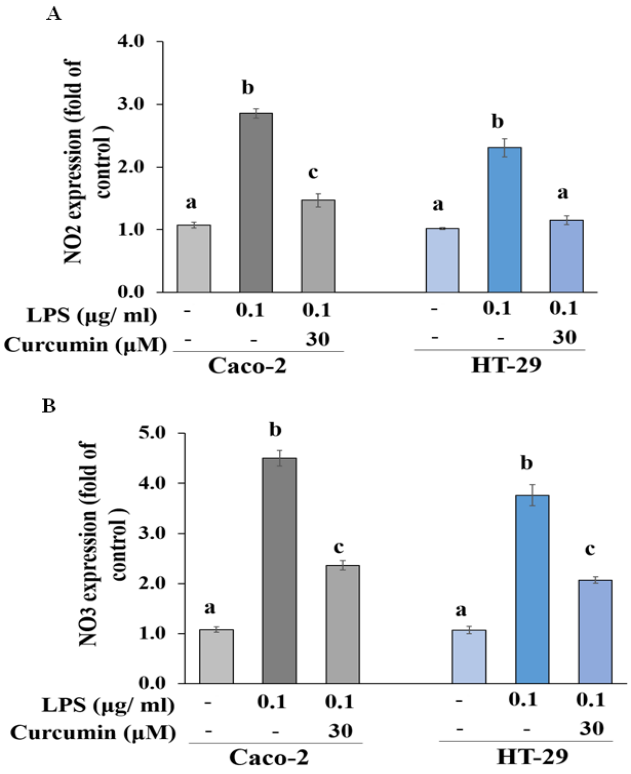

Figure 3. Inhibition of nitrite and nitrate in LPS-induced Caco-2 and HT-29 cells by curcumin. Nitrite and nitrate were detected from Caco-2 and HT-29cells cultured medium of each experimental group at 12hindividuallyby used Griess reagent(A-B).The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments

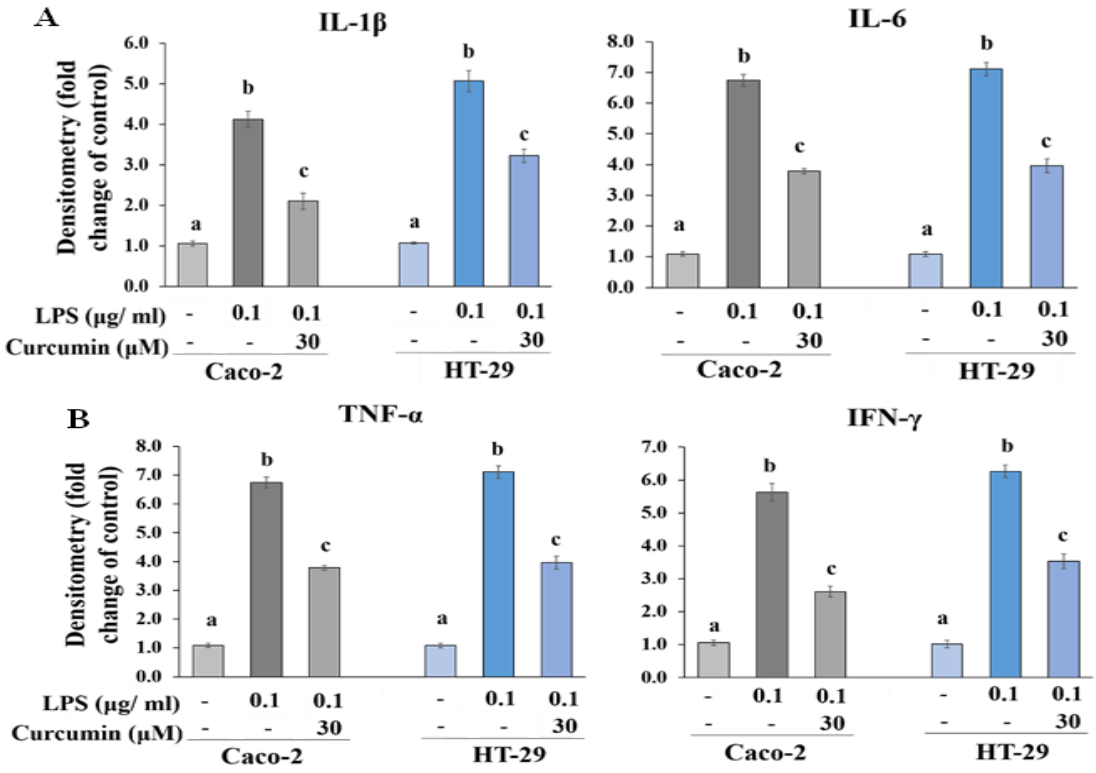

Figure 4. Cytokine expression in LPS-induced Caco-2 and HT-29 cells by curcumin. IL-1β, IL-6, TNF-αand IFN-γwere detected from Caco-2 and HT-29 cells cultured medium of each experimental group at 12hindividually (4A-B) by used microarray. The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments

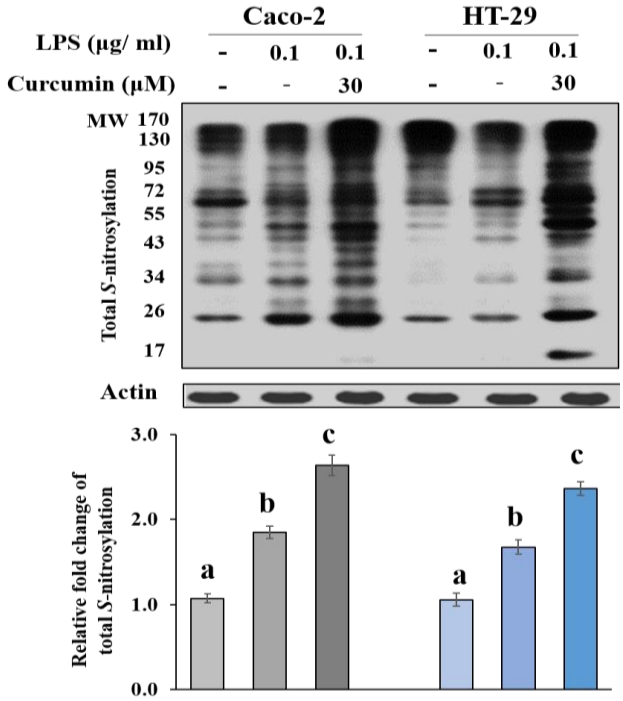

Figure 5. Total *S*-nitrosylated protein expression in LPS-induced Caco-2 and HT-29 cells by treated with curcumin. The total *S*-nitrosylated protein extracted from Caco-2 and HT-29 cell lysates of each experimental group at12h individually that was prepared by biotin switch method and detected by Western blot using anti-biotin. The bar-chart presented the relative quantification of total *S*-nitrosylation from Western blot. The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments

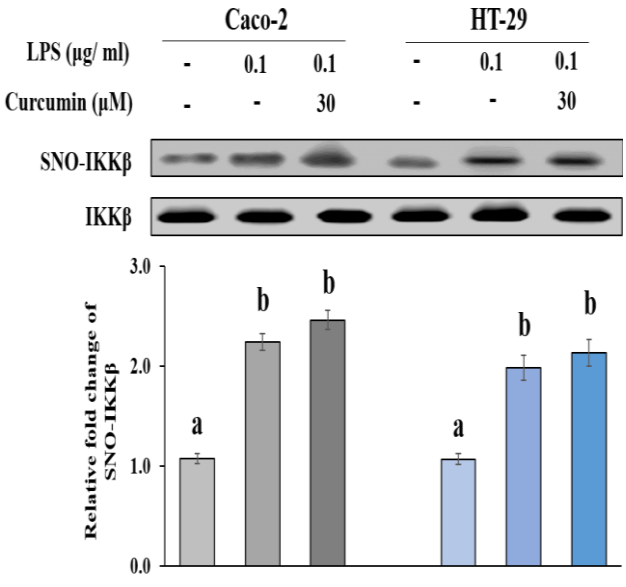

Figure 6. *S*-nitrosylated IKK-β Expression in LPS-induced Caco-2 and HT-29 cells by treated with curcumin. *S*-nitrosylated IKKβ as obtained from cell lysates of each experimental group at12hindividually by biotin switch method and detected by Western blot using anti-biotin. The expression level of IKKβ was as the control and relative fold change of *S*-nitrosylated IKKβ was normalized by IKKβ protein expression. The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments

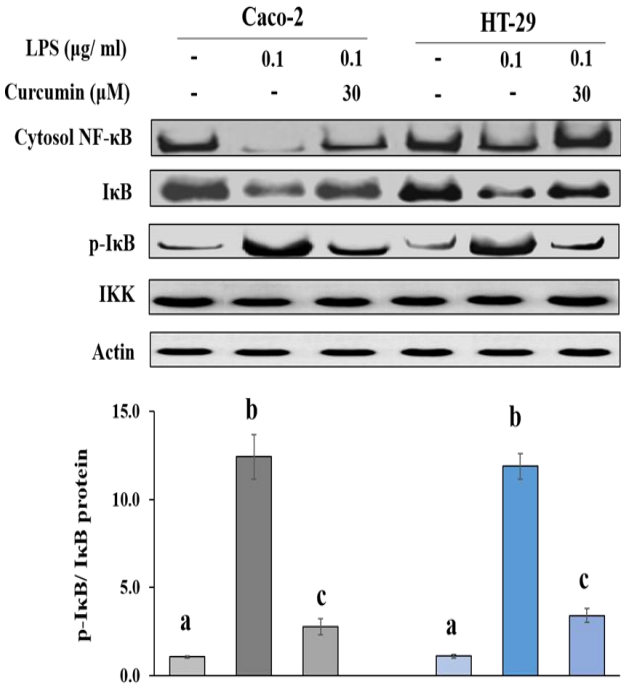

Figure7. IκBphosphorylation and NF-κB activation in LPS-induced Caco-2 and HT-29 cells by treated with curcumin. The expression level of phosphorylated IκB (pIκB), IκB, and cytosolic/nuclear NF-κB proteins were detected by Western blot. Actin expression was as the loading control. The bar chart presented the relative quantification of p-IκB expression normalized by IκB protein expression The data was repeated 3 times independently to ensure reproducibility and the standard deviation of the mean are represented as error bars (n=3). Values with different letters were significantly different ($p< 0.05$) at corresponding concentrations between different treatments

3.3. Curcumin Involves Protein *S*-nitrosylation

S-nitrosylation on cysteine residues of proteins is one of the targets and intermediates for free NO [32,33], here, we further use biotin switch method to detect whether curcumin can regulate the *S*-nitrosylated protein expression on LPS-treated Caco-2 and HT-29 cells. Western blot result showed that significant signal of *S*-nitrosylation on total proteins was increased at the group treated with curcumin (Figure 5), suggesting that the effect of curcumin may inhibit the NO oxidation to further maintain *S*-nitrosylation on proteins.

3.4. Curcumin Attenuated NF-κB Translocation by *S*-nitrosylayed IKK

Based on our previous result that curcumin can inhibit the expression of iNOS and pro-inflammatory cytokines and maintain protein *S*-nitrosylation, we further address whether curcumin-mediated protection from the inflamed human intestinal cells, Caco-2 and HT-29, was associated with alterations in NF-κB activation. As shown in Figure 6, *S*-nitrosylation on IKKβ was significantly increased both in LPS-induced human intestinal cells and curcumin-treated cells. The result indicated that curcumin maintained the *S*-nitrosylation on IKKβ in LPS-induced human intestinal cells. Consistent with these findings, phosphorylation of IκB, the downstream substrate of

IKKβ, was increased in LPS-induced human intestinal cells but repressed in curcumin-treated cells (Figure 7). The localization of NF-κB was also observed in nucleus by LPS activation and repressed in cytosol by curcumin. Taken together, the result indicated that curcumin might repress the IKKβ activity through *S*-nitrosylation and sequentially inhibit the phosphorylation of IκB, as well as attenuate the activation of NF-κB.

4. Discussion

Prior studies focused on the manner in which curcumin decreases inflammation on the activated immune system; however, the effect of curcumin on *S*-nitrosylation under inflammation was rarely addressed. In previous study, we found curcumin to possess anti-inflammatory properties in lipopolysaccharide (LPS)-induced macrophage cells due to the involvement of curcumin and *S*-nitrosylation in the NF-κB pathway [21]. Now, this study concerned inhibitory effects of curcumin on NF-κB pathway in two type of inflamed human intestinal cells, Caco-2 and HT-29. These results are also consistent with curcumin represses the activity of inhibitor-κB kinase in dextran sulfate sodium-induced colitis by *S*-nitrosylation [22].

S-nitrosylated IKK lost the ability to induce IκB from phosphorylation [34]. Curcumin maintained *S*-nitrosylated IKK with 30 μM at 12h in an *in vitro Inflamed Human Intestinal Mucosa Model* in our study, which showed curcumin might remain *S*-nitrosylation and caused the IKK to lose the ability for NF-κB activation. On the other hand, curcumin also reduced pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ from LPS-induced human intestinal cells. Furthermore, the cytokines caused iNOS production, and subsequent NF-κB activation was reduced. Consistent with previous studies, the pro-inflammatory cytokines were blocked by curcumin [35,36,37]. Therefore, our findings are crucial in the treatment of inflammatory conditions with curcumin, because curcumin reduced IκB phosphorylation by maintained *S*-nitrosylated IKK, and also reduced pro-inflammatory cytokines that cause iNOS expression, and subsequently eliminated the peroxide of NO to decrease the inflammation. There are several independent or interrelated mechanisms involve anti-inflammatory effect of curcumin have been reported [38,39,40]. However, the relationship of curcumin and *S*-nitrosylation provides insights into the mechanism of inhibition of NF-κB by phytochemicals, which have anti-inflammatory effects. This study is the first attempt to examine the role of inhibitor-κB kinase and nitric oxide on the anti-inflammation of curcumin via target protein *S*-nitrosylation in an *in vitro Inflamed Human Intestinal Mucosa Model*. The results would be helpful to future investigation into the application of anti-inflammatory bioactivities or nutritional ingredients. These basic findings will provide a potential yet new concept for both nutrition and biochemistry research, and the phytochemical of preventive medicine. In the future, we try to compare curcumin with a positive control such as a known anti-inflammation drug to check the efficiency and facilitate the mechanism investigation.

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